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HODGKIN'S DISEASE*

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CONTENTS

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Part III. Cytology and Etiology

Part IV. Therapy

INTRODUCTORY REMARKS

By Albert S Gordon

Department of Biology, Graduate School of Arts and Science, New York University, New York, N. Y

In the conception of this monograph it was felt that its prime purpose should be to cut across broad areas of the problem so as not only to include discussions of the etiologic and therapeutic aspects of the disease process itself, but also to project a detailed consideration, at a fundamental level, of [illegible]

the eosinophil, all of which receive attention in these pages

At the same time, it was felt that contributions from both academicians and clinicians might serve to cross-fertilize ideas and concepts that could ultimately lead to a better understanding of the disease, both with regard to *its causes and possible avenues of treatment* *It is also* hoped that investigators now concerned with fundamental problems relating to the lymphocyte and reticulum cell derivatives will be stimulated by this publication to apply their basic information and techniques to the solution of the vexing but challenging entity of Hodgkin's disease.

OUTLINE OF PROBLEMS IN HODGKIN'S DISEASE

By Antonio Rottino

Hodgkin's Disease Laboratory, St Vincent's Hospital, New York, N Y.

Although interest in Hodgkin's disease has always been great and contributions to the literature on the subject voluminous, progress has been exceed-
ately we now know little
Research in Hodgkin's
he discovery of the ameliorative effects of X rays and the mustard compounds, has been frustratingly sterile.

The chief purpose of this monograph is to stimulate those engaged in fundamental research into finding application of their work to the problem of Hodgkin's disease.

I shall therefore emphasize a few of the many questions in need of resolution First, there is the important one concerning the nature and cause of the disease, second, that relating to the function of the system primarily affected, namely, the lymphoreticuloendothelial system, and finally, that dealing with the function of the eosinophil.

Also involved are questions that are fundamentally genetic and immunologic in nature While I shall touch upon each of these questions here, they are considered in detail elsewhere in these pages

Some readers may not have any special interest in Hodgkin's disease per se, even though working in some capacity with the system vitally affected by it To reiterate, I hope their interest may become sufficiently engaged that, as they pursue their own particular studies aimed at elucidating the enigma of the lymphocyte, histiocyte, or eosinophil, they will bear Hodgkin's disease in mind and be alert to note anything applicable to its solution

Since I am a pathologist, it is logical and natural for me to begin with the histopathology of the disease Although the pathologist has a very good grasp of the histological changes and the sequence of their occurrence in the natural p , to
interpret the
small lym, ulls
also make an early appearance, but the reticuloendothelial cell is the one whose structure becomes altered drastically At first only a few of them are visibly affected, and they are inconspicuous and may even be overlooked

The changes that the reticuloendothelial (RE) cell undergoes consist of an increase in total size and in the size of its nucleus, which may assume very bizarre forms Two, three, or even more nuclei may persist in a cell Visible alterations occur in the chromatin and nucleolus, the latter may become very large in size and may be seen as two or more round or irregular bodies The cytoplasm of the cell may become unusually granular Nothing comparable to this is seen to occur either in the lymphocyte or in the eosinophil All this has led me to conclude (and this is not an original thought) that the target for

the causative agent of Hodgkin's disease is the RE cell, and that the proliferation of lymphocyte and eosinophil perhaps represents purposeful reaction on the part of the host.

As the disease progresses the number of affected reticulum cells increases. After a variable period of time, from one year to as many as ten or more years, the lymphocytes diminish in number and this is followed by a decrease in the number of Sternberg-Reed cells (abnormal histiocytic cells). All this is associated with concomitant increase in reticulum and collagen, and in the final stages we find the node relatively acellular and replaced by fibrous tissue In terminal cases fibrosis may affect every lymph node in the body and every follicle in the spleen. Strangely enough, the Kupffer cells in the liver are not affected, which indicates to me that they are of a different species from the RE cells in the spleen and lymph nodes The lymphoid system in the stomach is occasionally involved, but that of the small intestine almost never in the sense described above Instead, the lymphoid tissue in the intestine undergoes atrophy.

The one fact that emerges clearly from the study of the pathological process [illegible]

impairs and destroys this function?

Since there is a correlation between the sequence of the pathological changes and response to therapy (excellent while the nodes are predominately lymphocytic, and not at all when the nodes are universally fibrous) and since, also, death is the inevitable consequence of Hodgkin's disease, it is logical that we ask ourselves how much of all this can be attributed to the destruction of the lymphoid apparatus

One function of this apparatus is related to immune processes Although the poverty of the immunological mechanism in Hodgkin's disease has been discussed, no one has as yet given clear-cut evidence that this poverty is a fact Perhaps the methods described in this publication by J H Heller for measuring the function of the RE system may prove useful in correlating diminishing function with anatomical change

Another point merits discussion does the lymphoid system have subtle functions in addition to those connected with immunity? Rebuck and his collaborators, in their presentation, suggest that the lymphocyte is a storehouse for protein, in other words, that it is a cell analogous to the fat cell If this were true we should have a ready explanation for the gradual emaciation which develops late in the disease Our own studies (unpublished data) of the blood of Hodgkin's disease patients show a decrease in the albumin fraction only, whether this could be correlated to the lymph node changes, I do not know

Next, let us consider the ubiquitous eosinophil present in variable numbers throughout the disease The significance of this cell in the reaction of the host to the disease is something to which we should all like to have an answer

Some investigators interpret its presence to mean that there is an allergic component in Hodgkin's disease. This concept has not been substantiated thus far by factual data. Of interest is the belief expressed by R S Speirs in his paper that the eosinophil contributes to the antibody pool. Specific antibodies have not yet been demonstrated in Hodgkin's disease.

Of great importance in Hodgkin's disease are the Sternberg-Reed cells. In my opinion a key to the disease will possibly be found when the nature and the cause of the changes that are seen as the reticuloendothelial cell becomes transformed into the Sternberg-Reed cell are finally elucidated What is it that so profoundly changes the size of the cell and the size and shape of its nucleus? What is the significance of the large pools of nucleolar material? I look forward to the day when someone will isolate from these pools the causative factor of the disease. I hope that research such as that presented here by M. J. Kopac may be the approach for which we are looking

The search for the cause of Hodgkin's disease has engaged workers ever since it was first shown to be a disease entity. Many types of organism have been suspected, as discussed more fully elsewhere in these pages by R. B Kassel, W. L. Bostick, and A R. Reymers, whose presentations emphasize the possible role of a virus Since eventually it may be shown that a specific virus, as most of us understand the term, is not to be related etiologically to Hodgkin's disease, may I suggest other possible causative mechanisms? For one, it may be that the disease is triggered, not by a specific virus, but by a variety of viruses affecting the mucous membranes of the body. Should this be true, we should then have to search in the patient himself for the specific set of circumstances that permits Hodgkin's disease to develop in him and not in others What these circumstances are is the problem requiring research.

A second possibility is that Hodgkin's disease may be similar to disabilities such as glomerular nephritis or rheumatism, in which the body, in response to some infectious agent, produces an antibody that has a damaging effect upon a specific substrate in our case the nuclear material of the reticulum cell. Since genic material that normally replicates under controlled conditions is included in this substrate, under abnormal transformation and alteration caused by these hypothetical antibodies replication could become uncontrolled and the disease set into motion.

For completeness and for the information of those not acquainted with the subject, Hodgkin's disease is unique in that it attacks humans only. It has never been induced in animals, nor are there reports setting forth in clear-cut manner acceptable criteria for believing that the disease has ever occurred [illegible] The nonexistence in animals of a counterpart [illegible] the highly specific nature of the substrate [illegible] This high specificity is further evidenced by the fact that the human disease is rare, which indicates that susceptibility occurs in a highly select and rare group of about twenty per million[1] Very important questions arise concerning the factors involved in this specificity That genetic factors play a role is attested by the fact that not infrequently the disease occurs in several members of a family, as in mother and son, father and son, or aunt and nephew.

Of great interest and still unexplained is the higher incidence of Hodgkin's disease in the male, with a frequency of two to one as compared to the female. The disease obviously is not sex linked; nevertheless it may be that the hormonal milieu of the male provides an environment more conducive to its development than does the female hormonal pattern As yet there are no data suggesting that the factors involved favor the occurrence of disease more often in the male than in the female

To the clinician, the question of paramount importance is therapy; his chief preoccupation is the treatment of the patient Although X-ray therapy initially seemed to offer hope, time has shown the goal of cure to be still distant. Nitrogen mustard seemed to have great promise, but it, too, leaves much to be desired We have not found that X ray combined with the chemotherapeutic agents now available has increased longevity in these patients

In the period from 1943 through 1946 we treated 83 patients with X ray alone; 25, or 30 per cent, survived longer than 5 years. In the period 1946 through 1951 we treated 93 patients with X rays and chemotherapy combined. Thirty-two, or 35 per cent, survived beyond 5 years The chemical agents used during this latter period were nitrogen mustard and triethylene melamine (TEM). Although the series is admittedly small, there is no indication in our experience that chemotherapy alone or in combination with X ray has increased duration of life beyond the period resulting from use of X ray alone Chemotherapy, however, has contributed to the control of symptoms and to rendering the patient more comfortable, and to this extent it is a distinct

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to the work of the National Chemotherapy Committee, Bethesda, Md With the resources at the command of this body, something of value is bound to emerge This brings added interest to the presentation of Sidney Farber, Chairman of the Chemotherapy Committee Although Farber makes no announcement of a new miracle drug, I am encouraged by his enthusiasm and the application of his talents to the chemotherapy program.

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For completeness and for the information of those not acquainted with the subject, Hodgkin's disease is unique in that it attacks humans only. It has never been induced in animals, nor are there reports setting forth in clear-cut manner acceptable criteria for believing that the disease has ever occurred spontaneously in lower animals The nonexistence in animals of a counterpart of Hodgkin's disease must indicate the highly specific nature of the substrate necessary for the disease to occur This high specificity is further evidenced by the fact that the human disease is rare, which indicates that susceptibility occurs in a highly select and rare group of about twenty per million.[1] Very important questions arise concerning the factors involved in this specificity That genetic factors play a role is attested by the fact that not infrequently the disease occurs in several members of a family, as in mother and son, father and son, or aunt and nephew.

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Despite this, we can look forward with hope to the work of the National Chemotherapy Committee, Bethesda, Md With the resources at the command of this body, something of value is bound to emerge This brings added interest to the presentation of Sidney Farber, Chairman of the Chemotherapy Committee. Although Farber makes no announcement of a new miracle drug, I am encouraged by his enthusiasm and the application of his talents to the chemotherapy program.

I have presented a few of the major problems posed by Hodgkin's disease There are many more, but those that I have mentioned are well treated in this monograph Should it succeed in raising just a little of the fog surrounding just one problem this publication will have served its purpose.

Reference

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Part I. The Lymphocyte

POTENTIALITIES OF THE LYMPHOCYTE, WITH AN ADDITIONAL REFERENCE TO ITS DYSFUNCTION IN HODGKIN'S DISEASE

By J. W. Rebuck, R. W. Monto, E. A. Monaghan, and J. M. Riddle
Department of Laboratories and Department of Medicine, The Henry Ford Hospital, Detroit, Mich.

Introduction

The known potentialities of the lymphocytes are basically threefold: lymphocytopoietic, trophocytic, and defensive. Although listed separately, these functions are often interrelated.

The formation of lymphocytes by lymphocytes. Self-multiplication, or the formation of lymphocytes by lymphocytes, is a homoplastic type of lymphocytopoiesis consisting of repetitive formation of daughter lymphocytes. Downey and Weidenreich,[1] working with sections of animal lymph nodes and

in mitosis only in the thymus among the lymphoid tissues, and then but infrequently. Pulvertaft and Jayne[3] cultivated lymphocytes from a malignant exudate and found them capable of mitosis for over 1 week. Trowell[4] illustrated small lymphocytes in mitosis from his cultures of lymph nodes and more recently[5] reported occasional mitoses in large and medium lymphocytes even after 6 days in a synthetic medium. Moeschlin[6] depicted mitoses in small lymphocytes obtained from human spleens and has commented on their relative scarcity. Sundberg[7] observed mitoses in small lymphocytes in films of

possession of a cytocentrum complete with centrioles, a nucleolar apparatus, and mitochondria. The cytocentrum of the lymphocyte, first described by Weidenreich,[9] has been the subject of further study by Wallgren,[10] by Ackerman and Bellios,[11] and by Bessis.[12] The latter author, through microcinematography, found the cytocentrum to be a dense, relatively rigid element capable of deformation of the more plastic nucleus. The nucleus, thrust against the ... or less deeply indented

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by the dense chromatin masses of the mature lymphocyte in air-dried smears, is readily studied in sections or wet smears, as pointed out by Downey and

Weidenreich,[1] by Maximow,[13] and by Bloom.[14] Stockinger and Kellner[15] were able to bring out the nucleolus in smears by staining with methylene blue at pH 4 9, and supravitally they stained the lymphocytic nucleolus with brilliant cresyl blue Perry and Reynolds,[16] employing pyronin Y, found small amounts of pink material in the nucleus of adult lymphocytes Pulvertaft and Jayne[3] and Ackerman and Bellios[11] demonstrated the nucleolus of the lymphocyte with phase-contrast microscopy, and Rinehart[17] did so with the electron microscope Mitochondria, originally described in lymphocytes by Cowdry,[18] using a weak solution of Janus green B, have been found in mature lymphocytic cytoplasm with the phase-contrast microscope[11, 12] and by electron microscopy[12, 17] Elongated and spherical forms have been described.[11, 12 17-19] Identifying cristae are present in electron micrographs.[17]

Additional structural components of the lymphocytic cell body are: azurophil granules,[20] which may or may not be present in any given lymphocyte, several small bodies that stain deep red with neutral red,[21] an abortive endoplasmic reticulum (archoplasmic reticulum),[12] a large, refractile, spherical granule known as a Gall body,[22] and a Golgi zone.[23]

Trophocytic functions of the lymphocyte. The apparent disparity between the relative infrequency of mitoses in lymphocytes and the great growth potentials of lymphocytic tissues may now be resolved through a consideration of 2 further properties of lymphocytes first, their heteroplastic formation from reticulum cells, in addition to their formation as daughter cells of lymphocytes and, second, their possession of a long, true life span in contrast to their occasional short sojourn in the blood stream

In 1902, in her classical description of the cytology of Hodgkin's disease, Reed[24] called attention to the heteroplastic formation of lymphocytes as follows

"The lymphoid cells, which are so abundant in the young growth, are usually of the small, ordinary type. They are quite uniform in size and shape. No karyokinetic figures were ever observed in such cells. Lymphoid cells of a larger type are occasionally seen. We consider that the lymphoid cells arise from proliferation of the mother cells of the lymph nodes, and presumably also from the endothelial cells of the reticulum, which are analogous The great increase in the number of lymphoid cells in the early stages of the disease might explain, on a mechanical basis, the occasional relative increase in the number of these cells in the blood Such a phenomenon could not be constant, for as the fibrous tissue increases in the glands, as the disease progresses, the number of the lymphoid cells is diminished "

A decade later, Downey and Weidenreich[1] established our modern concepts of the reticuloendothelial origin of lymphocytes through painstaking sectional cytoanalyses of the lymphocytic tissues Their observations on this point may be summarized as reticulum cells → large lymphocytes → lymphocytes More recently Sundberg[7, 25] has correlated sectional studies with the newer hematological methods of imprint cytology, thereby expanding our knowledge of heteroplastic lymphocytopoiesis to embrace the following. undifferentiated reticular cell → hematopoietic reticular cell → reticular lymphocyte → imma-

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The formation of lymphocytes by lymphocytes. Self-multiplication, or the formation of lymphocytes by lymphocytes, is a homoplastic type of lymphocytopoiesis consisting of repetitive formation of daughter lymphocytes Downey and Weidenreich,[1] working with sections of animal lymph nodes and spleens, showed that mitoses were most numerous in large and medium-sized lymphocytes, and that dividing cells, while more numerous in germinal centers, could occur in any portion of the nodes Kindred[2] found small lymphocytes in mitosis only in the thymus among the lymphoid tissues, and then but infrequently Pulvertaft and Jayne[3] cultivated lymphocytes from a malignant exudate and found them capable of mitosis for over 1 week Trowell[4] illus- [illegible]

lymphocytes obtained from human spleens and has commented on their relative scarcity. Sundberg[7] observed mitoses in small lymphocytes in films of nonleukemic marrows from patients in all age ranges, and she concluded that all lymphocytic cell types are capable of mitoses, but that mitoses are relatively rare in small lymphocytes. Gowans[8] depicted mitotic figures in a small proportion of the large and medium lymphocytes in stained films of rat thoracic duct lymph, but he did not see them in small lymphocytes

The mitotic ability of the mature lymphocyte is further supported by its possession of a cytocentrum complete with centrioles, a nucleolar apparatus, and mitochondria The cytocentrum of the lymphocyte, first described by Weidenreich,[9] has been the subject of further study by Wallgren,[10] by Ackerman and Bellios,[11] and by Bessis.[12]

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cytocentrum during cellular movement, became more or less deeply indented Centrioles demonstrable by the iron hematoxylin stain were depicted both by Weidenreich[9] and by Wallgren [10] The nucleolar apparatus, usually masked by the dense chromatin masses of the mature lymphocyte in air-dried smears, is readily studied in sections or wet smears, as pointed out by Downey and

cyte survival of at least 21 days. The second slow decline suggested to Hamilton either the reutilization by lymphocytes of large fragments of the nucleic acids or nucleoproteins of their progenitors (see Gowans[9] and Trowell[31] above) or alternatively a second type of leukemic lymphocyte with a longer life than 85 days. Ehrlich and Seifter[33] proposed that lymphocyte breakdown not only furnishes building substances, but that the purines serve as phosphorus-transferring enzymes for synthetic processes.

Some insight has thus been gained for an understanding of the role of the entire mass of lymphocytes as a labile protein depot. Moreover, the large nucleic acid content of the lymphocytes suggested to Hamilton[34] that lymphocytes function as a store of nucleic acids, the nucleic acids of the lymphocytes serving additional functions as templates for the synthesis of protein

Defensive functions of the lymphocyte. The phagocytic ability of the lymphocyte, as a lymphocyte, is real, although scant. Tschaschin[36, 37] and Downey[38, 39] demonstrated that small lymphocytes phagocytosed vital dyes. Hertzog[40] incubated blood from human patients presenting absolute and relative lymphocytoses with *Staphylococcus aureus* and *Streptococcus viridans*. His photomicrographs of phagocytic lymphocytes are objective evidence of this phase of lymphocytic activity. Rebuck, Smith, and Margulis[41] and, more recently, Koszewski and his associates[42, 43] presented micrographs of phagocytic lymphocytes in preparations from man.

Lymphocytes are the most important blood-borne source of the macrophages, as demonstrated originally by Metchnikoff.[44, 45] Monocytes and the various histiocytes of the connective tissues are, of course, additional sources of macrophages, and the number of these cells available determines the importance of their contribution to the macrophages formed in an inflammatory area. Rebuck and Crowley[46] reviewed the literature pertaining to this function of the lymphocyte and, in addition, outlined a procedure in which the cellular exudate in single lesions in man was sampled hour by hour. Their photomicrographs depicted lymphocytes present as lymphocytes at 2 to 9 hours, at 9 to 14 hours many of the lymphocytes were hypertrophied, at 14 to 18 hours further lymphocytic hypertrophy eventuated in macrophage formation. Transformation of the lymphocytes of man into macrophages occurred with the following changes in the lymphocytes as such: the division of coarse chromatin into small pieces and increases in parachromatin, nuclear size, irregularity of nuclear membrane, chromatin-parachromatin distinction, and size of cell body.

Lymphocytes, as lymphocytes, form antibodies. Harris *et al.*,[47] Dougherty *et al.*,[48] and White and Dougherty[49] showed that cells of the antibody-forming tissues, predominantly lymphocytes, were rich in antibodies, whereupon an extensive literature arose (see Fagraeus[50] and Coons *et al.*[51]) confirming the original demonstration by Kolouch[52] that antibody formation is closely associated with plasma cell formation. Wesslen,[53] however, was able to show that lymphocytes from the thoracic duct of rabbits immunized with *Salmonella*

ture lymphocyte → lymphocyte. Three distinct experimental approaches now attest to the validity of this manner of lymphocytogenesis. First is the type experiment of Harris and Harris,[26] who transplanted thin slices of rabbit lymph node and spleen to the chorioallantoic membrane of 9-day-old embyronated eggs After 4 or more days of implantation increased numbers of reticulum cells showed all gradations to newly formed lymphocytes Second was the group of experiments performed by Mann and Higgins[27] and by Gowans[3] Yoffey[28] had reported that the output of lymphocytes from the thoracic duct of dogs could replace all the lymphocytes in the blood several times daily. The combined output of the 4 major lymphatic trunks of the rabbit was estimated by Hughes, May, and Widdicombe[29] to be capable of replacing the lymphocytes in the blood 11 times daily. A method for the continuous collection of thoracic duct lymph from unanesthetized rats devised by Bollman, Cain, and Grindlay[30] enabled Mann and Higgins[27] to establish the fact that the initial output of lymphocytes was indeed high, but that with prolonged drainage of lymph, the daily output of lymphocytes fell progressively to less than one third of their initial count. Mann and Higgins suggested that the loss of a small number of dividing cells necessary for producing lymphocytes might explain the dropping output. Gowans[3] was able to effect continuous reinfusion of living lymphocytes into unanesthetized rats with thoracic duct fistulae and thus to prevent the progressive fall in thoracic duct output. Cell-free lymph or killed lymphocytes were ineffectual This investigator suggested 3 ways in which the living lymphocytes maintained output from the thoracic duct (1) they could subsequently die in the lymph nodes, releasing substances for the production of new cells; (2) they might leave the blood and re-enter the thoracic duct via the tissue spaces or the lymph nodes; and (3) the lymph nodes might be repopulated by the 5 per cent of large and medium lymphocytes in the thoracic duct lymph that were depicted *in vitro* in mitosis In another type of experiment, Trowell[31] grew rat lymph nodes in a synthetic medium and found that after a few days the reticulum cells that still contained the pyknotic remains of phagocytosed small lymphocytes were in process of differentiation into new lymphocytes, thus providing a histological basis both for the reutilization concept and for heteroplastic lymphocytopoiesis.

Reminiscent of the delayed awareness of the long survival time of the human erythrocyte has been the progressive demonstration of the long life span of the lymphocyte Ottesen[32] claimed that by labeling desoxyribonucleic acid (DNA) with P^{32} the blood lymphocytes are composed of 2 groups, one with a mean blood span of 3 to 4 days, and the majority with a mean age of 100 to 200 days. Osgood and his associates[33] measured the incorporation of P^{32} into the DNA of the lymphocyte in chronic lymphocytic leukemia and found an average survival time of 84 days Hamilton[34] utilized C^{14}-labeled purines in his studies of nucleic acid synthesis in the lymphocytes in chronic lymphocytic leukemia, [illegible]

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Trowell[31] grew rat lymph nodes in a

Materials and Methods

Cells of the exudate in acute inflammation in man were studied with a previously reported[16] procedure by which serial preparations were obtained chronologically from a single lesion and stained in the same manner as blood smears or submitted to appropriate cytochemical analyses. In this procedure the epithelium is scraped away from a small area on the forearm. After inoculation with a suitable inflammatory excitant the lesion is covered by a cover slip. Cells of the inflammatory exudate migrate to the undersurface of the cover slip. When this has been accomplished (30 minutes to 1 hour) the cover slip is removed, air-dried, and stained. At the same time another cover slip is placed over the same lesion and the process is repeated at timed intervals throughout the cycle of inflammation.

Blood stains employed for permanent preparations were May-Grünwald-Giemsa, Wright-Giemsa, and Leishman's stains.

Cowdry's[62] method for the M. nadi oxidase reaction was employed. Fixation was over formalin vapor for 2 hours. In addition to the standard test, the oxidative activity was controlled with an identical run, except that 10^{-3} M KCN was added to the reagents as suggested by Brenner.[63] All the preparations were studied and photomicrographs made (FIGURES 1*a* and *b*) immediately after the completion of the tests, as the indophenol blue may fade. Washburn's modification[64] of the Goodpasture method was employed for study of leukocytic peroxidase activity, and May-Grünwald-Giemsa was the counterstain (FIGURES 1*a* and 2*a*–*d*).

The calcium phosphate method of Gomori[65] for nonspecific alkaline phosphatases (FIGURES 3*a* and *b*) was controlled by similar study of sections of human prostate. Gomori's[65] lead phosphate method for acid phosphatase (FIGURE 3*c*) was similarly controlled by application of the method to sections of human prostate. Fixation in both instances was in cold acetone for 1 hour.

The periodic acid-Schiff (PAS) sulfite leukofuchsin reaction[66] was studied in these preparations without digestion (FIGURE 1*d*) and after 1 hour of digestion at room temperature in a solution of buffered (phosphate) diastase of malt (FIGURE 1*e*). Fixation was for 1 hour in buffered formalin.

Sudanophilic changes were outlined by the Sudan black B-May-Grunwald-Giemsa method of Baillif and Kimbrough.[67] Fixation was in a mixture of formalin and 95 per cent ethyl alcohol as recommended by those authors (FIGURES 3*d* and *e*, and 4*a* and *b*).

The 42 lesions previously described[16] in healthy human volunteers, which consist of over 250 separate cover slip preparations taken at 1.75, 2, 3, 3.75, 5, 6, 7, 9, 10, 12, 14, 15, 16, 16.5, 18, 20, 21, 22, 23, 24, 28, 31, 40, and 47.5 hours of inflammation, served as controls for the investigations reported herewith. In the control groups (FIGURES 5*a* and *c*, and 6*a* and *c*), in addition to the trauma of the technique alone, egg white, diphtheria toxoid, triple typhoid vaccine, old tuberculin, and first-strength purified protein derivative (PPD) were employed as nonpyogenic antigens applied to the lesions in human subjects not systemically immunized to the respective antigens.

typhi or horse serum were capable of producing antibodies upon incubation, although the lymphocytes in the form in which they enter the blood stream did not contain them.

Lymphocytes are one of the important sources of plasma cells. With the decisive demonstration by Kolouch and his associates[54] that antibody formation was associated with reticulum cell formation of plasma cells in rabbit marrow and its subsequent extensive confirmation, the importance of lymphocytes as a source of plasma cells tended to be overlooked. Downey,[55] Maximow,[13] and Michels[56] all traced plasma cells to a lymphocytic origin, cognizant that they could arise from the mesenchymatous elements of the connective tissues, as well. The recent work of Roberts, Dixon, and Weigle[57] has gone far to resolve many of the apparently contradictory views regarding the cells responsible for antibody formation. Lymph node cells consisting of 90 per cent lymphocytes or peritoneal exudate cells consisting of 71 per cent macrophages obtained from donor rabbits previously immunized with bovine serum albumin were transferred by these workers subcutanously and intramuscularly to roentgen-irradiated, immunologically inert recipient rabbits The transfer of these cells caused a typical secondary response in the recipients after stimulation with the original antigen Serial biopsies of the transfer site in the recipients revealed classic plasma-cell formation by the transferred lymphocytes in the first group of experiments and plasma cell formation by the transferred macrophages in the second group, and the cytological observations were correlated

in response to the sensitizing antigen.

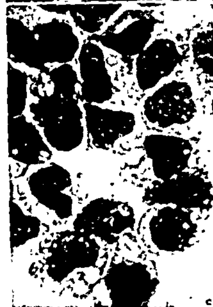
The transfer of cutaneous hypersensitivity by the lymphocyte. When Chase[59] succeeded in transferring hypersensitivity to tuberculin by means of viable cells from lymph nodes of tuberculous guinea pigs, the reactivity was thought to be correlated with the amount of transferred lymphocytes. Working with

and Rebuck[46] demonstrated lymphocytes → macrophages in a large series of patients. By harvesting large numbers of macrophages that had been formed by the lymphocytes on the cover slip preparations of patients exhibiting hypersensitivity to tuberculin or tularin and transferring the macrophages to control subjects, they were able to show that the ability of the lymphocyte to transfer cutaneous hypersensitivity is also transmitted to the macrophage it forms

It is the purpose of this paper to explore the chain of cytochemical events by which the transformation of lymphocyte to macrophage is accomplished in acute inflammation in man and to determine the nature of the failure of effective leukocytic defense in Hodgkin's disease.

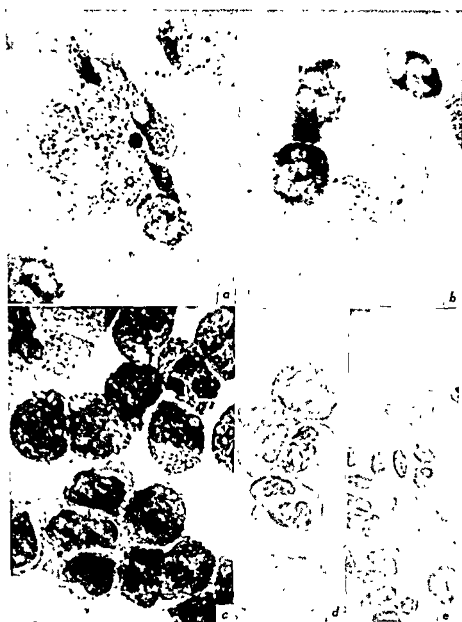








trol lesion in *c* at the left. ×1275.


a
b
c
d
e

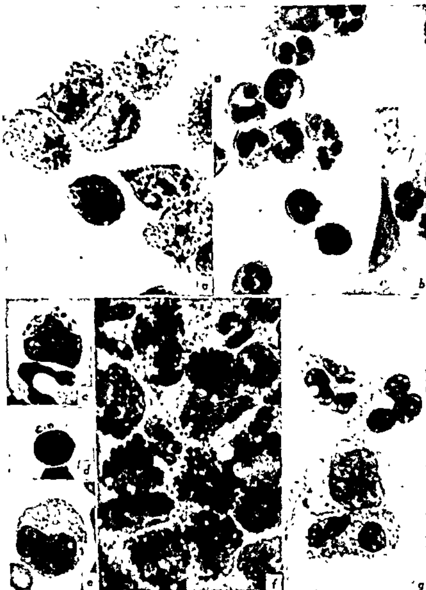
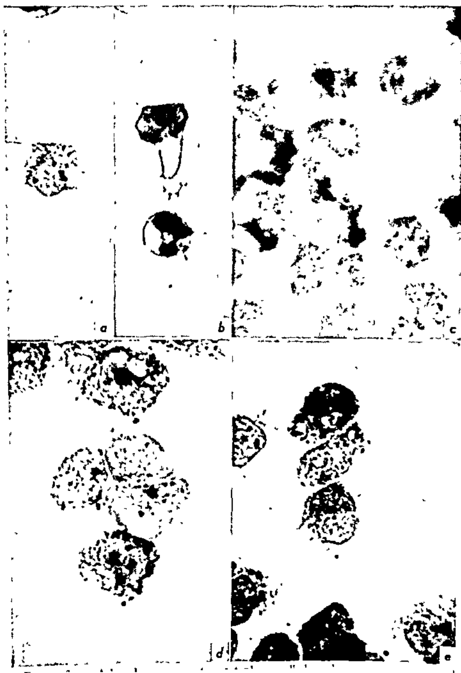
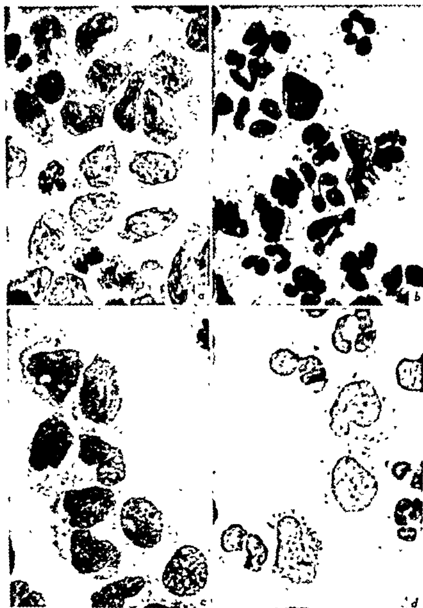


FIGURE 1. (a) [illegible]


a
b
c
d
e



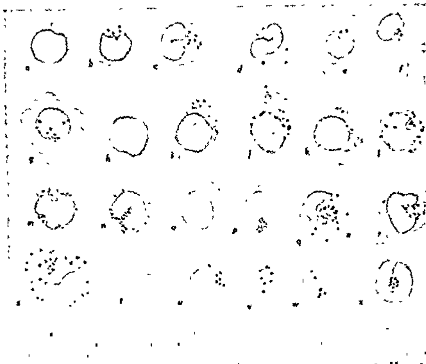
[illegible] FIGURE 5a [illegible] Compare with *c* control at the left


a


b


c


d



slips had been previously prepared by applying suitable quantities of neutral red in absolute ethyl alcohol to their surfaces from which the alcohol was allowed to evaporate Edges of the cover slips were sealed with paraffin, and the preparations were studied in warm-stage enclosures at 37 5° C Exudative cells from 4 blisters in 3 subjects were so studied (FIGURES 7 and 8)

Results

Acute inflammation in man controls Cover-slip preparations from single lesions in the skin of the forearm and stained with ordinary bloodstains served as controls. For a detailed description the reader is referred to the previous report of Rebuck and Crowley[46] Four of many stages from one such lesion

The major portion of the present work comprised a study of 135 separate cover slip preparations in 25 different lesions as follows: the M. nadi reaction was studied at 2.5, 4, 6, 7, 7.5, 9, 10, 12, 13, 14, 15.5, 16, 22, 24, and 29.5 hours of inflammation in 3 lesions in control subjects; peroxidase activity was studied at 3, 5, 8, 9.5, 10, 12, 14, 14.5, 18, 27, and 34 hours of inflammation in 3 lesions in control subjects and at 3, 6, 9, 12, 13, 23.5, and 27 hours of inflammation in 2 lesions in patients suffering from the granulomatous stage of Hodgkin's disease; alkaline phosphatase activity was studied at 3.5, 7, 10, 12, 14, 25, and 26 hours of inflammation in a lesion in a control subject; acid phosphatase activity was studied at 3.5, 10, 12, 14, 25, and 26 hours of inflammation in a lesion in a control subject; glycogen content was ascertained at 6, 12, and 24 hours in 2 lesions in a control subject; sudanophilic properties of the leukocytes were studied at 3, 6, 9, 12.5, 14, and 21 hours in 2 lesions in 2 control subjects and at 3, 5, 7, 9, 12, 14, 24, 29, and 32 hours in 2 lesions in a patient suffering with chronic lymphocytic leukemia

Phagocytic activity of the lymphocytes was studied in 2 bone marrow aspirates from patients suffering from kala-azar and histoplasmosis respectively (FIGURES 4c and *d*). In addition, serial preparations studied at 3, 5, 7, 10, 12, and 24 hours in a lesion in a control subject were tested for phagocytic activity by the addition of a small amount of India ink to the lesion at the seventh hour (FIGURE 4*f*)

The response to nonpyogenic antigens was studied at 3, 4, 5.5, 6, 7, 8.5, 9, 11.5, 12, 13, 14, 22.5, 23.5, 24, 24.5, and 27 hours of inflammation in 9 lesions in 5 patients with the granulomatous stage of Hodgkin's disease (FIGURES 2*b* and *d*, 5*b* and *d*, and 6*b* and *d*) Four of the patients had been previously treated with lymphocytolitic agents (steroids, nitrogen mustard, or roentgen the[illegible]

in [illegible]
undenatured pertussis antigen Sodium acid urate served as the excitant in the phosphatase studies

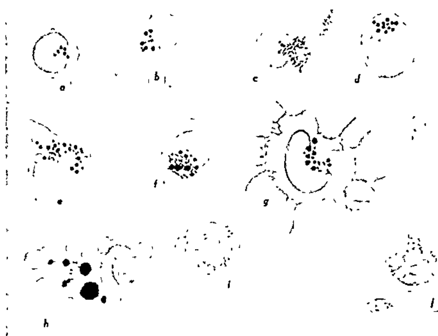
arm of human volunteer controls as follows a mixture of powdered Chinese cantharides and petrolatum was applied to a circular area of skin measuring about 1.5 cm in diameter This mixture was covered with adhesive tape so as to exclude air In about 7 hours a blister forms in the area with 0.5 cm elevation of the epidermis At this same time 0.05 ml of sterile egg white [illegible]

syringe. [illegible]
blister fluid. A few drops served to prepare supravital preparations Cover

of small fragments shed into the exudative fluids. FIGURE 4*e*, taken at the same magnification, illustrates a monocyte of the peripheral blood for structural and size comparison. At 14 hours of inflammation (FIGURE 6*b*) in the same lesion, the lymphocytes have gained another micron in diameter, the increase being largely cytoplasmic in nature. In addition to the superimposition of colorless organelles over the nuclear chromatin, there appears to be a true increase in parachromatin distinction At 24 hours in the same lesion (FIGURE 6*c*) the field is flooded with still larger mononuclears with the characteristic [illegible] rophages or histiocytes, the resultant

[illegible] *in inflammation in man* By the twelfth hour of inflammation the small exudative lymphocytes showed enhanced activity in the form of as many as 60 blue, cytoplasmic granules after treatment with nadi reagent. There is general agreement in the literature that lymphocytes in the peripheral blood are relatively deficient in this activity, a finding confirmed by us for the lymphocytes in the blood of the subject whose lesion is depicted here. The positive granules in the 12-hour lymphocytes (FIGURES 1*a* and *b*) were ordinarily distributed irregularly throughout the cytoplasm The blue granules were at times concentrated about cytoplasmic vacuoles and occasionally in clumps Neutrophils were strongly positive at 12 hours At later stages hypertrophied lymphocytes and macrophages were moderately positive. The addition of 10^{-3} M KCN to the standard test resulted in complete absence of the blue granules in the lymphocytes, of their larger forms, and of the neutrophils of the exudative preparations

Peroxidase in the lymphocytes of man in acute inflammation At 2 75 hours of inflammation the rare lymphocyte observed showed a negatively reacting cell body At 5 hours the predominant cells were neutrophils, and their cytoplasm was filled with dark blue granules A few tissue macrophages and lymphocytes were present, and these latter cell types now contained fine, blue-staining granules in their cytoplasm At 9 5 hours and 10 hours the neutrophilic leukocytes stained as before for peroxidase A small number of round free cytoplasmic fragments were packed with peroxidase-positive granules Almost one fourth of the cells were now lymphocytes Most of the lymphocytes (FIGURE 2*a*) contained numerous peroxidase-positive granules diffusely distributed throughout their cell bodies An occasional lymphocyte was devoid of positive granules or contained only 2 or 3 of them Tissue macrophages contained numerous positive blue granules Sometimes it appeared that a phagocytosed neutrophilic cytoplasmic bud within the lymphocytic cytoplasm had contributed a circumscribed area of peroxidase positive granules to the cytoplasm of the lymphocyte (see cell in lower right corner of FIGURE 2*a*) At 12 hours of inflammation (FIGURE 1*c*) the slightly larger lymphocytes present numerous blue granules in their cytoplasm At 14 5 hours (FIGURE 2*c*) further lymphocytic hypertrophy revealed some diminution in the positivity of their reaction The tissue macrophages and occasional monocytes showed a similar degree of positivity The positivity of the gradually enlarging mononuclears persisted at the 17- and 20-hour stages The cells depicted in FIGURES



(*h*) Macrophage at the 23 hour, 15 minute stage of inflammation (experiment H-19) (*i* and *j*) Neutrophils with elongated pseudopodia from the inflammatory exudate All ×2000

in which the inflammatory excitant was 0.05 ml 1:1000 tuberculin (O.T.) are depicted in FIGURES 5*a* and *c* and 6*a* and *c* They will serve as reference points for the cytochemical studies described below and as comparative exudates for the abnormal responses elicited in the various diseased states studied. At 9 hours (FIGURE 5*a*), a considerable number of lymphocytes were found among the numerous neutrophils in the lesion Except for their irregular outlines, a feature of all exudative cells arrested in their natural activities, the lymphocytes appear unchanged with the ordinary bloodstains used here and measure about 10 μ in diameter At 12 hours (FIGURE 5*c*) in the same lesion the lymphocytes are more numerous and slightly larger (12 μ in diameter) Their cell bodies are larger and show an increase of basophilic substance and colorless material The latter unstained portions appear to represent negative images of unstained organelles and were referred to as an increase in hyaloplasm in our previous reports Such unstained colorless flecks overlie the lympho-

described for FIGURES 5*a* and *c* and 6*a* and *b* in the control series, and for FIGURES 1*c* and 2*a* and *c* in the series stained for peroxidase activity. In 1 of 2 lesions studied serially in a patient suffering from chronic lymphocytic leukemia a definite depression of sudanophilia was observed FIGURE 4*b*, taken from this patient at 12 hours of inflammation, depicts 2 lymphocytes, a macrophage, and several neutrophils almost devoid of sudanophilic constituents. This exudate should be compared with the 3 preceding exudates obtained from a control subject and similarly stained.

Increased phagocytic powers of the lymphocytes in inflammation. As outlined in the discussion above, the phagocytic potentialities of the lymphocyte, as a lymphocyte, although poor, are real FIGURE 4*c* depicts *Leishmania donovani* phagocytosed by a medium lymphocyte, and FIGURE 4*d* depicts *Histoplasma capsulatum* phagocytosed by a small lymphocyte from the marrow of a patient so infected. FIGURE 4*e* at the same magnification as FIGURES 4*c* and *d*, illustrates a monocyte with an ingested erythrocyte taken from the blood of a patient suffering from a drug-induced pancytopenia. The true monocyte from the blood of man possesses a larger cell body and a larger nucleus than any of the lymphocytes described in this paper Moreover, the monocytic nucleus is lobulated and possesses a distinct threadlike chromatin pattern FIGURES 1*c* through 6*d* were taken at the same magnification (×1275), hence the monocyte in FIGURE 4*e* may be used as a reference cell for comparison with the other exudative cells. FIGURE 4*f*, taken at 10 hours of inflammation in a control subject, illustrates the marked increase in phagocytic ability attained by small and medium lymphocytes after even a short exposure to the inflammatory environment Three hours prior to the study in FIGURE 4*f* a sterile bacterial loop had been touched to India ink then applied to the lesion The increased phagocytic ability parallels the aforementioned cytochemical changes and increases in proportion to the cytoplasmic growth of the lymphocytes in these preparations

Leukocytic cycles in acute inflammation in man as modified in the granulomatous stage of Hodgkin's disease * Typical of the series of preparations obtained from 9 lesions in 5 patients in the granulomatous stage of Hodgkin's disease is the cycle of changes depicted in FIGURES 5*b* and 6*b* and *d* from our Case 1 This patient, a white female aged 28, was suffering from a severe, generalized form of granulomatous Hodgkin's disease of 2 5 years' duration Four months prior to study she had received a course of nitrogen mustard therapy, and at the time of study she was receiving both adrenocorticotropic hormone (ACTH) and prednisone The white blood cells numbered 18,450, with 79 per cent neutrophils, 18 per cent lymphocytes, and 3 per cent monocytes Two lesions were studied At 3 hours of inflammation, a few edematous neutrophils were found At 6 hours numerous neutrophils were observed and a few macrophages had made their appearance At 9 hours of inflammation in the same lesions (FIGURE 5*b*) moderate numbers of swollen neutrophils, some macrophages, and a rare monocyte were observed, but the lesion was lacking in lymphocytes (com-

* We are indebted to Michael Brennan, Division of Oncology, The Henry Ford Hospital, for permission to study three of these patients

1c and 2a and c) were from different cover-slip preparations from the same lesion, 0 05 ml. diphtheria toxoid serving as the inflammatory excitant. These cells were counterstained with May-Grunwald-Giemsa and, therefore, can serve as examples of the progressive over-all changes undergone by the lymphocytes, they are also illustrative of the peroxidase positivity attained by lymphocytes in acute inflammation in man.

PAS (periodic acid-Schiff) reaction in lymphocytes in inflammation. Preparations from 2 lesions were studied. In the first series the cover slips were stained after preliminary fixation in buffered formalin without prior digestion. At the important 12-hour stage of inflammation (FIGURE 1d) both lymphocytes *and neutrophils show a granular PAS positivity. At the same 12-hour stage* in the second control lesion (FIGURE 1e) whose preparations were subjected to prior diastase digestion, it is seen that almost all PAS reactivity has been deleted in lymphocytes and neutrophils

Nonspecific alkaline phosphatases in lymphocytes in inflammation. A series of preparations from a single lesion was studied with Gomori's[65] calcium phosphate method. FIGURES 3a and b show lymphocytes from the tenth hour of acute inflammation Both cells show the black areas indicative of enzymatic activity in their cytoplasm A nonspecific nuclear localization of the precipitate is also visualized.

Acid phosphatases in lymphocytes in inflammation in man. An additional series of preparations from a single lesion was followed with Gomori's[65] lead phosphate method for acid phosphatase. The precipitated lead salt of the liberated phosphate is visible as transformed black lead sulfide in FIGURE 3c. The 4 lymphocytes, the monocyte, and the 2 macrophages depicted amid the neutrophils in this figure present a nuclear localization for the activity noted. The exudate was obtained from the twelfth hour of inflammation in man.

Lymphocytes in acute inflammation after staining with Sudan black B and May-Grunwald-Giemsa. Following the exposure of the exudative cells of the cover-slip preparations (obtained from 2 lesions in a control subject) to Sudan black B according to the method of Baillif and Kimbrough[67] striking changes

appearance in the lymphocytic cytoplasm at the side of and overlying the nucleus. Many of these small staining bodies may be seen clustered about the cytocentrum of the medium-sized lymphocyte in FIGURE 3d At 12 5 hours

cytoplasmic areas ... of such ... depicted in FIGURE 4a these black staining bodies are not only he cell body, but are so concentrated about sette of Sudan-stainable bodies As in the peroxidase studies, these cells were also counterstained with May-Grunwald-Giemsa and are confirmatory of the progressive lymphocytic transformation

studied in our Case 5, that of a 15-year-old white female suffering from far-advanced Hodgkin's granuloma of 1 year's duration. This patient had been the recipient of extensive roentgen therapy to the left thorax, mid-dorsal spine, and right cervical area, and of a long course of chlorambucil* (600 mg. total dosage). During the period of our study she received roentgen therapy to her neck. Her white cells numbered 13,200/cu mm at this time, but consisted of only 5 per cent lymphocytes and 95 per cent neutrophils As in Case 2 there was almost complete absence of peroxidase activity noted at the 12- and 27-hour stages. The lymphocytic hiatus was again present, as it was in all 9 lesions studied in this group

Direct and continuous observation of living lymphocytes from acute inflammation in man; application of supravital techniques † Four blisters were prepared in the arms of the 3 control subjects as described above in the section *Materials and Methods*. It should be noted that at the time the blister had formed (7 to 7.5 hours after application of cantharides) 0 05 ml of sterile egg white and 0 05 ml of 1:10,000 neutral red in physiological saline were injected into the blister. In this way the exudative mononuclear cells were at all times exposed to neutral red. Any exudative cell functionally capable of segregating neutral red was thus afforded full opportunity to do so *in vivo* The addition of egg white to the blister fluid brought forth increased numbers of exudative mononuclear cells of all types

At 10 75 hours, 12 5 hours, and 23.5 hours after application of the cantharides, aspirations of fluid were made from the same blister Supravital preparations were studied immediately in 2 or 3 separate warm-stage enclosures after each aspiration

From the 7.5-hour aspirate, numerous motile neutrophils were obtained. Inasmuch as they have been well described previously as to motility and specific granular uptake of neutral red, we devoted most of our attention to a study of the agranular leukocytes and tissue cell types present in the preparations Neutrophils were frequently found with extremely tenuous processes such as those depicted in FIGURES 8*i* and *j* The actual process by which the cytoplasmic portion containing specific granules was shed into the exudative fluid was never observed by us However, numerous free portions of neutrophilic cytoplasm (FIGURES 7*i* to *k* and 8*c*), were observed in these preparations At this stage the exudative mononuclears were of 2 types lymphocytes and macrophages. The lymphocyte depicted in FIGURE 7*a* was drawn from this preparation It possessed scant cytoplasm, a round nucleus, and no neutral red vacuoles The same cell is depicted in FIGURE 7*b* as it appeared 15 minutes later A few neutral red vacuoles are present, and a portion of the nuclear membrane exhibits marked activity. The same cell 30 minutes after it was first observed is depicted in FIGURE 7*c*. A deep indentation had appeared in the nucleus, 9 neutral red vacuoles had appeared, and the cytoplasm was more abundant.

* Chlorambucil is *p* (di-2-chloroethyl) aminophenylbutyric acid, a cancerolytic drug

† The first of 4 lesions so studied was reported by the senior author (J W Rebuck) in a thesis submitted to Hal Downey, The Graduate School, University of Minnesota, Minneapolis, Minn, in 1947

pare the lymphocytic infiltrate at this stage in FIGURE 5a). The over-all cellularity was numerically inferior to that of the controls At 12 hours (not illustrated) the scant exudate in these lesions consisted of a mixture of neutrophils and macrophages, and again lymphocytes were rare in the lesion. At 14 hours in the same lesions (FIGURE 6b) the exudate was again scant and consisted of moderately sparse numbers of neutrophils and some macrophages, lymphocytes were absent. This lymphocytic deletion is the more striking when compared with the 14-hour stage of controls as shown in FIGURES 2c, 4a, or 6a. At 24 hours in the same lesions in Case 1 the defective exudate persisted (FIGURE 6d), consisting only of focal collections of now somewhat larger macrophages and an unusual preponderance of neutrophils (compare with the complete macrophage response in control FIGURE 6c at 24 hours).

FIGURE 5d, which depicts 11 5 hours of inflammation in Case 3, has been interposed for the same stage in Case 1 because the 33-year-old white female represented in Case 3, although diagnosed by cervical lymph node biopsy 5 months before our study as suffering from the granulomatous stage of Hodgkin's disease, had received no therapy This patient's white blood cell count some time prior to our study was 6000/cu mm , with 59 per cent neutrophils, 33 per cent lymphocytes, 6 monocytes, and 2 eosinophils Her early exudative cells again consisted of a few swollen neutrophils At 11 5 hours (FIGURE 5d) the sparse exudate consisted largely of macrophages with a few neutrophils and lymphocytes interspersed The lymphocytic fault is striking when compared with similar control stages in FIGURE 1a and c or 3e, and 5c. This hiatus in lymphocytic participation in the leukocytic cycles as observed in Hodgkin's disease bore a striking resemblance to the near absence of lymphocytes in similar lesions studied in a patient with true agammaglobulinemia (FIGURE 4g) At 22 5 hours numerous macrophages were found, a few lymphocytes and neutrophils were also present

FIGURES 2b and d are from exudates obtained from 1 of 2 lesions studied in our Case 2 This case was that of a 22-year-old white female with Hodgkin's granuloma dating to 13 months prior to study One day prior to our study this patient began her first treatment, roentgen therapy to her mediastinum At the time of study her white cells numbered 11,000, cu mm , consisting of 88 per cent neutrophils, 7 per cent lymphocytes, 1 per cent each of basophils and eosinophils, and 3 per cent monocytes A study of peroxidase activity in the exudative cells of this patient revealed almost complete suppression of the reaction FIGURE 2b, taken at 9 hours of inflammation, reveals some of the numerous neutrophils and macrophages present in this lesion together with an occasional monocyte However, peroxidase activity is almost completely lacking This figure should be compared with FIGURE 2a, which shows the strong positive peroxidase reaction obtained in a control lesion at the same stage. At 13 hours of inflammation in the same lesion in Case 2, macrophages were numerous (FIGURE 2d) and some edematous neutrophils were found, and the suppression of peroxidase activity (compare FIGURE 2c, a similar positive reactivity in a control lesion) persisted as it did through 23 5 hours of inflammation in this lesion. Peroxidase activity was further tested for in 1 of 2 lesions

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FIGURES 7e and f depict another lymphocyte in active motion, presenting the "hand-mirror" shape with an active anterior cytoplasmic area and a passive cytoplasmic tail. The neutral red vacuoles, few in number, are tucked into a slight indentation of the posterior aspect of the nucleus. FIGURES 7d and g, from the same preparation, depict typical macrophages with large, blunt pseudopodia, little migratory activity, and few to many scattered neutral red vacuoles.

From the 10.75 hours' aspiration both depolarized (rounded) and polarized (migratory) lymphocytes were obtained, as well as phagocytic histogenous macrophages The lymphocyte depicted in FIGURE 7h was first observed at 11 hours and 20 minutes of inflammation At 11 hours and 39 minutes this same lymphocyte had come in contact with a free bud of neutrophilic cytoplasm that contained both specific granules (red) and a few phagocytosed particles of egg white (blue), as shown in FIGURE 7i. The lymphocyte cytoplasm at this time also contained 3 particles of egg white within vacuoles and two neutral red vacuoles. At 11 hours and 40 minutes the neutrophilic cytoplasmic bud had broken free from the lymphocyte, but in so doing had separated into a smaller and a larger portion Six minutes later, at 11 hours and 46 minutes, this same lymphocyte (FIGURE 7k) was again in contact with the 2 neutrophilic cytoplasmic buds. Suddenly the larger of the 2 buds was repelled from the lymphocyte We are not certain as to whether the smaller bud was ingested *in toto* by the lymphocyte. In any event 5 minutes later at 11 hours and 51 minutes (FIGURE 7l) this lymphocyte now possessed more than 15 neutral red vacuoles, as well as several ingested particles of egg white At 12 hours and 5 minutes it appeared as in FIGURE 7m One minute later the neutral red vacuoles, greatly increased in number, had formed a rosettelike arrangement about the cytocentrum in the region of the now deeply cleft nucleus of this cell However, this cell, only 46 minutes previously, had been the small lymphocyte depicted in FIGURE 7h.

Under continuous direct observation, this lymphocyte had not only exhibited its phagocytic ability by the ingestion of the foreign protein (egg white) and portions of the neutrophilic cytoplasmic bud, but it had elaborated a great number of neutral red vacuoles that arranged themselves about the cytocentrum The cytocentrum itself was in the neighborhood of a deep cleft that had appeared in the lymphocyte nucleus In the 46 minutes in [illegible]

an ocular micrometer

FIGURE 7o, from this same preparation, depicts another lymphocyte that was depolarized, and just a few microns to the left of the lymphocyte depicted in FIGURE 7h The lymphocyte depicted in *o* of the same figure during the 46 minutes of observation had no contact with neutrophilic cytoplasmic buds, yet 46 minutes later it too had elaborated a rosette of neutral red vacuoles (FIGURE 7p)

FIGURE 7q depicts a macrophage from the same preparation The macrophages have now assumed the phagocytic function, as evidenced by several

ingested particles (blue) of egg white within their abundant cytoplasm. Neutral red vacuoles are more numerous and likewise have arranged themselves in the form of a rosette about the cytocentrum

neutral red vacuoles were arranged in the form of a rosette about their cytocentra Macrophages, one of which is illustrated in FIGURE 7*s*, now present a dispersion of their neutral red vacuoles. Marked irregularity of the nucleus of these histogenous macrophages is a nearly constant feature as depicted in FIGURES 7*d*, *q*, and *s*.

A few lymphocytes were still obtained from the aspirate of 23 5 hours, but most of the cells were hypertrophied forms The lymphocyte of FIGURE 7*t* at 23 hours and 45 minutes was migrating, as depicted Eight minutes later it had settled down, rounded up, and increased in size to become the cell shown in FIGURE 7*u*. From time to time lymphocytes that exhibited no functional or structural transformation were held under observation for varying periods. FIGURES 7*v* and *w* depict such a lymphocyte with drawings 7 minutes apart. The lymphoid cells are somewhat larger at this stage as shown in FIGURE 7*x* and 8*a*, and they show an increasing tendency to a rosette arrangement of the

supravital "monocyte." These cells would migrate in the "hand-mirror" shape characteristic of lymphocytes (as depicted in FIGURES 7*f* and 8*b*) However, from time to time, long, slender, pointed pseudopodia would dart out from the active anterior psuedopodial area of such lymphocytes and would be rapidly withdrawn in a manner highly suggestive of the pseudopodial form and action of the typical supravital "monocytes" depicted in FIGURE 8*g* Another of these hypertrophied lymphoid forms is depicted in FIGURE 8*d*

However, many of the cells at this stage are larger macrophage forms exhibiting the characteristics of supravital "monocytes" (FIGURES 8*c*, *e* to *g*) or macrophages (8*h*) The cell shown in FIGURE 8*g* exhibited under direct observation all the characteristics typical of a large supravital "monocyte" There was constant undulation of the cell border and, at the same time, numerous thin, delicate processes were thrown out as pseudopodia in all directions, from all sides The cell body was large, the nucleus was kidney shaped, and the neutral red vacuoles had aggregated in the region of the nuclear bay. The cell shown in FIGURE 8*h*, on the other hand, is a typical macrophage with almost no migratory activity. It possesses larger, blunt, more sluggish pseudopodia, large vacuoles, and dispersed, giant neutral red bodies

A review of FIGURES 7 and 8 depicting cells observed at various stages in our type experiment reveals that as the inflammation grew older the exudative

mononuclear cells, which at first were lymphocytes, gradually hypertrophied until the macrophage stage was reached As they did so their neutral red rosette of vacuoles became larger and more conspicuous until finally, when the large scavenger-cell stage was attained, the rosette once more was dispersed. Not depicted is the gradual change observed in types of locomotion Lymphocytes possess a "hand-mirror" type of locomotion, and the hypertrophied forms retain the lymphocytic type of locomotion but, in addition, present the long, slender, darting pseudopodia of "monocytes" Eventually the constantly undulating, multipseudopodial activity of the supravital monocyte is attained This in turn merges into the sluggish, blunt-pseudopodial activity of the macrophage

Three more blister preparations were similarly studied, and continuous observation of additional lymphocytes yielded similar transformations. It soon became apparent that those lymphocytes with definite undulatory movements of their nuclear membranes, no matter how scant their cytoplasm, were the most promising candidates for functional change and structural growth as described above.

Discussion

In the simplest cycle of acute inflammation in man, when the exciting antigen is a nonpyogenic one to which the control subject is not systemically immunized, migration of lymphocytes is prominent by the ninth hour (FIGURE 5a). At from 9 to 14 hours the lymphocytes increase in number and undergo

parachromatin in the enlarging nucleus An increase in the irregularity of the nuclear membrane also accompanies lymphocytic hypertrophy At 14 to 18 hours further lymphocytic hypertrophy eventuates in macrophage formation. Structurally, then, the majority of lymphocytes in the system we have used

Cytochemical analyses of the transformational process were rewarding Within a short time after arrival at the inflammatory site there was a gradual acquisition by the lymphocytes of oxidase (FIGURES 1a and b) and peroxidase

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findings ... usually missing in the lymphocytes as they are transported by the blood[67, 71] Only acid phosphatase[72-75] and glycogen,[71, 76-79] as depicted in FIGURES 1d and e and 3c in the lymphocytes of inflammatory sites have also been reported as being present to varying degrees in the lymphocytes of the blood Further-

more, the changes from relatively inactive to cytochemically active lymphocytes occurred at the key stages just prior to and during lymphocytic hypertrophy. Townsend and Campbell,[80] using Kolouch's[81] technique to study the inflammatory leukocytic cycles in the mouse after whole-body irradiation, found that the hypertrophying lymphocyte (their intermediate polyblast of lymphocytic origin) was the most radiosensitive of the inflammatory cells, the lymphocytes and macrophages showing no apparent structural changes in the inflammatory connective tissue spreads. Pertinent to these cytochemical changes in the lymphocyte in inflammation as it transforms into the macrophage are the recent observations of Sieracki* who, working in our laboratory, has devised a permanent mitochondrial stain for the exudative cells of man. Modifying the original Fain-Wolfe[82] technique, he found that as the lymphocytes became hypertrophied there was a relative and absolute increase in mitochondria. Most of the increased numbers of small and granular mitochondria were observed near the nuclear indentation or scattered above the nucleus. Dubreuil[83] was among the first to correlate granular mitochondrial changes in demonstrating the derivation of the mononuclears from lymphocytes. More recently Felix and Dalton,[84] following the transformation of lymphocytes to macrophages in the stimulated peritoneal fluids of mice, also observed that the mitochondria became finer and more numerous. Employing phase microscopy, these workers were able to follow thinning of the nuclear membrane as its uneveness in the lymphocytic stage became smoother with increasing cell growth. They also noted that, as the transformation to macrophage occurred, there was nucleolar enlargement and that the chromatin masses became less dense.

Since Sehrt's[85] report of parallel destruction of sudanophilia and indophenol oxidase activity it has become questionable whether enzymatic action is actually involved in the stable reaction.[42, 85] Gomori[85] believed that the formation of indophenol blue in the M nadi reaction could be explained for the granulocytic series by the presence of fat peroxides. Lillie and Burtner,[86] however, after a lengthy series of comparative testing of oxidase, peroxidase, and sudanophilic activities of the leukocytes concluded that it was improbable that leukocytic oxidase was a fatty acid peroxide. We have observed the destructive action of 10^{-4} M KCN on the activity in question in the neutrophils, in the lymphocytes, and in their transformational products in our exudative preparation. Although KCN has also been reported as destructive of blood granulocytic sudanophilia[37] the sudanophilia of leukocytes itself bears further study because of demonstrable differences in reaction from those of true fats and lipoids.[86]

The enzymatic status of leukocytic peroxidase is less open to question. Agner[87] isolated a green enzyme with characteristic absorption bands and peroxidase effect from leukocyte-rich material. Again, however, Gomori[85] cautioned that the reaction obtained histochemically may be due to a nonprotein catalyst as well as an enzyme. If the presence of peroxidase in leukocytes is established, its function therein is not as yet clarified. Agner[87] and Chance[89]

* Unpublished data.

both noted the similarity of the absorption spectrum of verdoperoxidase and cytochromes of Type A, but the latter author has also demonstrated that they are not identical.

The question of endogenous or exogenous sources of leukocytic oxidases and peroxidases raised originally by Wallbach[53] after injecting horseradish and extracts of leukocytes into the subcutaneous tissues of mice is of interest in that positive granular material in the exudative lymphocytes of our preparations, although usually distributed irregularly, was at times concentrated about cytoplasmic vacuoles and occasionally in dense clumps, suggesting an alternative origin through lymphocytic ingestion of positively reacting neutrophilic,

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terial observed activity in the lymphocytes in foci of chronic inflammation. The cytoplasmic activity noted in the lymphocytes of our preparations (especially FIGURE 3a) confirms this. The artifactual nature of nuclear localization in this method has been stressed by both Gomori[65] and Wachstein.[71]

Haight and Rossiter[73] deduced that the acid phosphatase activity of leukocytes is chiefly in the lymphocytes Rabinovitch and Andreucci[72] presented

region of

blood cells

round cell

ould have

stained by diffusion. Wachstein[71] also questions nuclear localization for this activity. Weiss and Fawcett[91] and Goldstein and McCormick[92] have reported cytoplasmic acquisition of reactivity of monocytes as they transformed into

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calized in the region of the centrosphere and Golgi apparatus, coinciding in pattern with that of the saliva-resistant PAS-positive material. They further suggested that, since macrophages segregate ingested foreign material in this region, the acid phosphatase found there may participate in the digestion of the phagocytosed material It is interesting in this connection to note that the PAS-positive material in the lymphocyte at the 12-hour stage of inflammation in man (FIGURE 1d) does not resist digestion (FIGURE 1e)

As mentioned above, many workers have been struck by the similarity of

There appeared to be a striking correlation between the mitochondrial pattern of these cells as observed by Sieracki[93] and the increase in lymphocytic sudanophilic cytoplasmic particles found in our preparations

In this discussion we have been particularly concerned with the acquisition by the inflammatory lymphocytes of properties not readily demonstrable in the lymphocytes in the blood. It should be emphasized that the study of lymphocytic structure and cytochemistry in the lymphocyte-forming tissues may also yield evidence of activities not manifest in the lymphocytes of the blood. For example, Graff[93] found that lymphocytes of the follicles of spleen and lymph node in animals and man were nadi positive, and Ackerman and his associates[74] have shown a small number of PAS-positive and sudanophilic granules and weak alkaline and acid phosphatase activity in the lymphocytes of nonneoplastic human lymph nodes

In view of the gradual depletion of the lymphocytes in the lymphocytopoietic tissues as described by Reed[24] above and the progressive lymphopenia observed by most investigators,[94] our finding of near lymphocytic depletion of the leukocytic cycles in Hodgkin's granuloma was not unexpected (FIGURE 2*b* and *d*, 5*b* and *d*, 6*b* and *d*). Although 4 of the 5 patients studied in this series were under treatment with lymphocytolytic agents, the lymphocytic hiatus in their exudative response did not differ with the different therapies nor from the same lymphocytic depletion in the untreated patient (FIGURE 5*d*) Similar lymphocytic abeyance has been observed in the exudative lesions in man after systemic ACTH[95] and topical cortisone,[96] and in hypogammaglobulinemia[98] (FIGURE 4*g*). Although the destructive effects of roentgen rays for lymphocytes has been well documented,[99-101] their effects, when studied on the inflammatory leukocytic cells of the mouse and the rabbit,[80] showed an over-all decrease in the number of infiltrating cells, the hypertrophying lymphocyte at 12 hours being the most radiosensitive Ackerman and his associates[102] noted marked diminution of phosphatase activity, ribonucleic acid (RNA), and the reaction for sulfhydryl in the lymphocytes of lymph nodes from rats after total-body X radiation The depression of peroxidase activity (FIGURE

been few reports of cytochemical analyses of the lymphocytes in this condition

When one considers that lymphocytic transformation to the macrophage is preceded and or accompanied by the long list of structural and chemical changes discussed above and summarized below, the failure of a given experimental system[104] to yield transformation of continuously observed individual lymphocytes may be appreciated When one further considers that the

the deleterious circumstances of the inflammatory environment in which the originally poor-bodied lymphocyte finds itself may well militate against lymphocytic transformation or even survival itself Under the conditions described above we have observed the living, individual lymphocytes as they progressively transformed into small histiocytes or macrophages, assuming a rosette of neutral red bodies, increasing cytoplasmic size, and ingesting foreign material into their

cell bodies as depicted in FIGURE 7a to c, h to n, o to q, t and u). Single cells, lymphocytes by every criterion, have been seen to enlarge, to undergo structural reorganization, and to change bodily into small phagocytic histiocytes or supravital monocytes, and they have done so while under continuous observation

Summary

A review of the literature concerning lymphocytic potentialities reveals 3 basic although interrelated functions· lymphocytopoietic, trophocytic, and defensive.

Lymphocytes can be formed by lymphocytes through their mitotic ability Lymphocytes possess nucleoli, centrioles, and mitochondria as key cellular organelles

The trophocytic function necessitates an understanding of the role played by the entire lymphocytic mass as a labile protein and nucleic acid depot The first property in this connection possessed by lymphocytes is their ability to be formed heteroplastically from mesenchymatous elements (reticulum cells), as well as from other lymphocytes This has now been demonstrated experimentally by the transfer of lymphocyte-forming reticulum cells to the chorioallantoic membrane of the chick and by the growth of lymph node in tissue cultures It is supported by the observation that failure of the lymphatic trunks to deliver their lymphocytes to the blood stream results in a progressive fall in lymphocytic output Studies of nucleic acid synthesis of the lymphocytes have shown a long life span for these cells

The defensive functions of lymphocytes are phagocytic and antibody-forming, both as lymphocytes and as the macrophages and plasma cells into which they are capable of transforming

The present studies have shown that transformation of lymphocytes into macrophages in acute inflammation in man was accompanied by the following functional and structural changes in lymphocytes as such increase in cytoplasmic size, increase in phagocytic ability, assumption of a positive peroxidase reaction, assumption of a positive nadi reaction, assumption of alkaline phosphatase activity, manifestation of constantly increasing sudanophilic cytoplasmic constituents, an absolute increase in mitochondria, presence of PAS-positive cytoplasmic material, presence of nuclear acid phosphatase, an increase in nuclear size, an increase in nucleolar size, an increase in irregularity of the nuclear membrane followed by a thinning and smoothing of the nuclear membrane, a division of the coarse chromatin masses into fine, angular pieces, and an increase in chromatin-parachromatin definition In addition, individual living lymphocytes from stimulated cantharides blisters were continuously observed as they transformed into small histiocytes or macrophages, assuming rosette of neutral red bodies, increasing cytoplasmic size, and ingesting foreign material

In nine lesions studied in five patients with Hodgkin's granuloma there was quantitative depression of the leukocytic infiltrate and almost complete depletion of lymphocytic participation in the inflammatory leukocytic cycles, a depression of leukocytic peroxidase activity was also observed.

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CONTROL AND FUNCTIONS OF THE LYMPHOCYTE*

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Previous studies on the utilization of purines and pyrimidines for polynucleotide synthesis in human leukemic leukocytes have shown differences in the pattern of ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) turnover of leukemic granulocytes and lymphocytes[15, 16] In granulocytes, RNA and DNA turnovers appear to be almost identical; this is the pattern of such rapidly dividing tissues as bone marrow and intestine. Consequently, myeloid tissue is assumed to be rapidly dividing In contrast, in lymphocytes the turnover of DNA is much less than that of RNA This relationship of DNA to RNA is seen in slowly dividing tissues such as resting liver, lymphatic tissue, taken as a whole, thus appears to be slow dividing Information on the survival time of leukocytes, derived from analyses of DNA turnovers, is in accord with this idea. If the assumption is made that DNA, once formed in a cell, persists essentially unchanged for the life of that cell, the average survival time of that cell can be calculated from the DNA-turnover data. Such calculations [illegible]

As noted earlier,[17] these assumptions are subject to limitations when applied to leukocytes Nevertheless, with these reservations, we have made calculations as others have done "Average survival time" comprises the time from the origin of the leukocyte in bone marrow or lymphatic tissue to its demise. The objectives and assumptions underlying these studies on the incorporation of purines and pyrimidines into the nucleic acids of leukocytes have been detailed elsewhere[16, 17] Analysis of data on the incorporation of adenine-8-C^{14} into the DNA of leukocytes of a patient with a leukemoid reaction gives 9 days as a first approximation for the average survival time of polymorphonuclear leukocytes The corresponding calculations on the DNA of leukemic polymorphonuclear leukocytes show that they survive about 23 days Interpretation of the lymphocyte DNA data is complicated by the decline, in two phases, of radioactivity in lymphocyte DNA first, an initial rapid decline, then a slow decline having a very long half time Calculations from the DNA of lymphocytes in chronic lymphatic leukemia before the slow decline show an average survival time of 85 days Some approximate calculations on data from the results of double-labeling following administration of C^{14} adenine and C^{14} orotic acid suggest that the survival time of lymphocytes is shorter in other patients with chronic lymphatic leukemia.

If 9 days is the survival time of the normal polymorphonuclear leukocyte in man, as indicated by Ottesen's data[32] as well as by my own,[17] and the av-

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phatic ducts of unanesthetized rats, Mann and Higgins[24] found that the lymphocyte count from thoracic or intestinal lymph fistulae fell progressively over a 5- to 6-day period, accompanied by decreased circulating lymphocytes in the blood. This decrease in output was independent of the rate of lymph flow (providing free continous flow was obtained), and was not affected by starvation, dehydration, or sham operations These investigators were unable to maintain lymphocyte output by the reinjection of fresh lymph, but the poor condition of their reinjected rats compared to that of controls indicates that their lymphocytes were moribund. If lymphocytes are long lived cells that recirculate, extensive losses via thoracic duct drainage would necessarily be followed by a lymphopenia.

Gowans[14] confirmed the findings of Mann and Higgins,[24] and by careful handling of lymphocytes prevented the fall in lymphocyte output upon returning to the animal all its lymph and lymphocytes Gowans developed an apparatus to reinfuse lymph and lymphocytes intravenously at approximately the rate at which they had flowed from the thoracic duct. The viability of reinfused lymphocytes was judged by their mobility *in vivo*. In contrast, lymphocytes damaged by ultraviolet irradiation or by heating to 56° or 45° C (treatment that apparently resulted in their delayed death within 7 hours when incubated *in vitro*) or cell-free lymph did not prevent the fall in output. We conclude that entrance of living lymphocytes into the blood is essential for maintaining the output of lymphocytes from the thoracic duct. The reinfused lymphocytes presumably keep thoracic output up by leaving the blood and returning to lymph nodes or lymphatic vessels The simplest interpretation of these results is that lymphocytes recirculate.

Another line of important evidence helps resolve the apparent contradiction between the short blood span calculated from thoracic duct cannulation data and the longer life span calculated from isotope data. Let us consider the total mass of lymphoid tissue and lymphocytes in the body, the ratio of blood lymphocytes to thoracic duct output, and daily production in relation to average survival times The calculations here presented are not novel in the past other investigators[28-41] have made similar calculations, but these have not been widely accepted because they have often been shrouded with details concealing their merit Recently, Patt[33, 34] straightforwardly discussed the myeloid-erythroid balance in man, this stimulated me to make similar calculations on lymphocytes in several species These calculations are tentative However, the order of magnitude of the results seems reasonably consistent with current knowledge and it provides, as discussed below, a possible solution to the problem of how long lymphocytes survive

To start, Patt's calculations[33] on erythropoiesis in man give figures for red cell production based on a well-established survival time. In a 70-kg man with a blood volume of 7 5 per cent and erythrocytes 5.5×10^9 cells/ml, the total erythrocytes in circulation are 29×10^{12} Since the average survival time of the erythrocytes is about 120 days, erythrocyte production must equal 24×10^{10} cells per day, that is, 10×10^9 cells per hour From the cells produced per hour, and knowing the mitotic time in hours and the number of mi-

erage survival time of leukocytes of both cell types is 13 days,[16, 23, 33] the normal lymphocyte clearly has a survival time greater than 13 days By rough calculation, assuming that lymphocytes constitute one third of the leukocytes, their survival time must be at least 21 days Ottesen,[32] who studied the incorporation of P^{32} into the DNA of both cell types, suggested that some lymphocytes have a short survival time (3 to 4 days), and others a longer time (100 to 200 days); in one patient 22 per cent of lymphocytes were short lived and 78 per cent long lived; in the other, 11 per cent short lived and 89 per cent long lived.

Evidently lymphocytes, whether normal and leukemic, live much longer than previously suspected; Janeway[21] stated, "So far as is known the usual fate of normal lymphocytes in man is to survive a very few hours." The idea that lymphocytes are short-lived cells have been based largely on lymphocyte counts and measurements of flow in the major lymphatic channels of the body, all with the tacit assumption that lymphocytes do not recirculate [42] From the very large numbers of lymphocytes that regularly enter the blood by the thoracic duct and the obvious conclusion, that since their number in the blood remains fairly constant they must be disappearing from the blood at the same rate, average life spans of lymphocytes of between 2 to 12 hours, depending on the species, have been calculated [1, 35, 38, 40] This line of reasoning fails to distinguish between the time spent by the lymphocyte intravascularly and the true life span (or, as I prefer to call it, the average survival time) of the lymphocyte True life span includes the time during which the maturing cell undergoes an unknown number of divisions in lymphatic tissue, plus the actual time spent intravascularly, as well as any time spent outside the vascular bed Nevertheless, the observation that in most animals the thoracic duct output of lymphocytes is sufficient to replace all the lymphocytes in the blood several times daily[41] has prevented this distinction from being drawn even by those[42] aware of the necessity In the rat, for example, Gowans[14] estimated that blood lymphocytes were replaced about 11 times daily by the output from the thoracic duct alone Since lymphocytes enter the blood by other routes, this represents a minimum figure of the daily replacement rate For the dog, Yoffey gives a minimal "daily replacement factor" of 2 06, and about 4 0 for the guinea pig [41] In the rabbit, Hughes *et al* [20] estimated that the lymphocytes in the blood were replaced about 11 times daily if the outputs of thoracic, cervical, subclavian, and right lymphatic ducts were combined [illegible]

span [38]

How are we to reconcile the thoracic duct cannulation data that suggest that lymphocytes survive for only a few hours and the isotope data that suggest that lymphocytes are relatively longer lived cells?

If we accept the figure of 14×10^{11} lymphocytes in the 70-kg man, and assume an average survival time of approximately 21 days, then the production of lymphocytes would be 6.5×10^{10} cells daily. This is approximately 6 times the number circulating in the blood at any one time, and a little over 3 times the daily thoracic duct output. This does not imply that 21 days is the established life span of the normal lymphocyte in man, however, our calculations from isotope data indicate that the life span of the lymphocyte must be of this order rather than hours. Nevertheless, if indeed we accept the idea that the lymphocytes in the peripheral blood are only a very small proportion

the mean body weight of 27 rats was 172 gm. and the blood volume was 8.6 ml. With a lymphocyte count of 8×10^3/cu mm, the total number of lymphocytes in the circulation was 68.8×10^6, and thoracic duct output 768×10^6 cells/24 hours[14] On the basis of 1 per cent lymphatic tissue, the 172-gm. rat has a total lymphocyte count of 3.5×10^9 cells, this is to be compared with the circulating lymphocytes 7×10^7 cells and a thoracic duct output of 7.7×10^8/24 hours

Taking 3.5×10^9 cells as the total lymphocytes in the rat, we can calculate the daily lymphocyte production, assuming a survival time of about 16 days, derived from nucleic acid turnover of all lymphoid tissue exclusive of the thymus[3] Thus, with this survival time, daily lymphocyte production is 2.2×10^8 cells, which is to be compared with 7×10^7 circulating lymphocytes and a thoracic duct output of 7.7×10^8 cells/24 hours Daily lymphocyte production in the rat comes out as about one third that of thoracic duct output, but three times greater than the number of lymphocytes in peripheral blood

I have made similar calculations on Yoffey's 400-gm guinea pig[42] with a blood volume 7.5 per cent of the body and a lymphocyte count of 4730/cu mm In the guinea pig, lymphocytes in the circulation totaled 142×10^6 cells and thoracic duct output was 480×10^6 cells/24 hours Assuming again that the guinea pig has 1 per cent lymphoid tissue, total lymphocytes in the body would be 8×10^9 cells, which compares with 14×10^7 cells in the circulation, a thoracic duct output of 48×10^7 cells per 24 hours, and a total of 3×10^9 lymphocytes that Yoffey has calculated are in the marrow of a 400-gm guinea pig Similarly, we can calculate from a total lymphocyte count of 8×10^9 cells the daily production of lymphocytes in the guinea pig with the arbitrary assumption that they have approximately the same survival time as lymphocytes in the rat, that is, 16 days With this assumption, daily lymphocyte production would be 5×10^8 cells, which may be compared with 1.4×10^8 lymphocytes in the circulation and 4.8×10^9 cells/24 hours of thoracic duct output Thus it would appear that daily production in the guinea pig approximates thoracic duct output

One can calculate the lymphocytes in man and the relation of the blood lymphocytes to this figure in another way Osgood[29] estimated that there were 3 gm of lymphocytes in the circulating blood Outside the blood, in lymphoid tissue, there are approximately 100 gm of lymphocytes (Osgood)[29] and 70

toses per precursor cell, we can calculate the number of precursor cells in the marrow as follows:

$$\text{Number of precursor cells in marrow } (N) = \text{cells produced per hour } (P) \times \frac{\text{mitotic time in hours } (t)}{\text{mitoses per precursor cell } (M)} \quad (1)$$

According to Japa,[22] the mitotic index for the erythroid series in marrow is 3.3 per cent, and from general data 45 minutes seems a reasonable assumption for the time of mitosis. Thus,

$$N = \frac{(10 \times 10^9)(0.75)}{0.033} = 230 \times 10^9 \text{ nucleated erythroid cells in marrow} \quad (2)$$

Similar calculations can be made for myelopoiesis in man. Since the distribution of mitosis in myeloid and erythroid tissue was 45 to 55, it can be estimated that granulocyte and erythroid production will be in the same proportion if the time of mitosis is the same in both series. The number of granulocytes produced per hour will thus be 8.2 × 10⁹ cells. The number of precursor cells in the marrow may be calculated as follows:

$$N = \frac{(8.2 \times 10^9)(0.75)}{0.01} = 615 \times 10^9 \text{ myeloid cells in marrow} \quad (3)$$

It will be seen that the number of myeloid cells, 615 × 10⁹, compared with nucleated erythroid cells, 230 × 10⁹, gives a reasonable $M:E$ ratio. The total polymorphonuclear production per day is 200 × 10⁹ cells. Taking the average survival time to be 9 days for the granulocyte (this includes 3 to 4 days maturation time in the marrow), the total of polymorphonuclear cells at any one time in the body equals 1000 to 1200 × 10⁹ cells. In the circulation at any one time there are about 23 × 10⁹ cells, that is, there are roughly 50 times as many granulocytes in the body as there are in the peripheral circulation. These calculations are consistent with leukophoresis in man[8] and the size of the vascular pools, for example, in the lungs.[6] Assuming the number of granulocytes in the periphery to be 50 times more numerous than those in the peripheral blood, we can calculate the proportion of lymphocytes in the peripheral blood to total lymphatic tissue. All lymphatic tissue in the body contains large numbers of lymphocytes, and it is almost impossible to survey a tissue section under high-power magnification without encountering one or two lymphocytes per field. There have been various estimations of the total amount of lymphoid tissue. Andreasen[2] gives extensive data for rats and estimates total lymphoid tissue to be between 0.5 to 1 per cent of total body weight. Yoffey[42] considers it possible that total lymphoid tissue is about 1 per cent of body weight. If we accept 1 per cent of body weight as a reasonable

Osgood[29]

erefore, the

mately one

This figure is to be compared with the 11 × 10[illegible] cells circulating in the peripheral blood at any one time and with a daily thoracic output of 16.8 × 10⁹ cells in 24 hours

ments of lymphocyte nucleic acids or nucleoproteins mediated by phagocytosis Trowell reviews these observations in more detail elsewhere in this monograph.

The intimations of reutilization of lymphocytes provoke one to speculate how their nucleic acids function. If nucleic acids provide templates for the construction of different proteins, it would be reasonable to expect that the presence of much nucleic acid in lymphatic tissue, which is an important site of protein-antibody synthesis, implies the same role in these cells. Nucleic acids in lymphocytes would thus serve as templates for making antibodies and, presumably, each of the numerous nucleic acids might make a different antibody.

Despite our qualms, this hypothesis accounts for transfer of immune reactions by the transfer of lymph nodes or cells from lymph nodes Both the hypersensitivity[10] and homograft[7, 27] responses after cell transfer are too rapid to be caused by the transfer of the original antigen with the cells. Since anti- [illegible]

body with time in the recipient,[11, 13] it is likely that some self-replicating mechanism has been transferred. If we accept a function of lymphocytes to be that of depots of specific nucleic acids for production of antibodies, these observations can be readily interpreted as a transfer and reutilization of viable templates

The reutilization hypothesis would thus make less mysterious the appearance of antibody long after limited contact between tissue and antigen. Whatever the explanation, there is, as Medawar has pointed out, an " extraordinary feat of memory embodied in the secondary or 'anamnestic' response."[27]

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gm in bone marrow; the latter figure is extrapolated from the guinea pig (Yoffey's data)[41] to man. Osgood,[29] on the basis of 10 lymphocytes per oil-immersion field, suggested a further 1300 gm of lymphocytes in the rest of the body. If we are very conservative and take one tenth of Osgood's figure (that is, one cell per oil-immersion field), this gives 130 gm. of lymphocytes throughout the tissues. That the total of lymphocytes outside the blood would be 300 gm. as compared with 3 gm in the blood (that is, 100 lymphocytes outside the blood to every 1 within it) appears to be a conservative calculation. All this is assumption, and all these figues are very uncertain, but one can use the formulas for erythroid and myeloid production to calculate the percentage of mitosis in lymphoid precursor cells. From calculations for the rat, mitosis in all lymphoid tissue comes out to 0.1 per cent at any one time This seems a reasonable figure in the light of Trowell's observations (personal communication) [illegible]
1 per [illegible] r
cent r [illegible]
[illegible]

how one may have large thoracic duct outputs and much lymphocyte production and still have lymphocyte survival consonant with isotope data.

The Reutilization Hypothesis

What does the prolonged retention of radioactivity following the administration of C^{14} adenine and C^{14} orotic acid in all four bases of the nucleic acids of lymphocytes mean?[16, 17] In our calculations on the survival time of lymphocytes this part of the curves has been ignored It is conceivable that the slow decline (with a half time of nearly one year) represents a second type of leukemic lymphocyte living much longer than our estimated 85 days Or, might this slow decline represent the reutilization by lymphocytes of large fragments of the nucleic acids or nucleoproteins of their predecessors? Present evidence, even with all four bases labeled and their radioactivity declining in parallel, does not permit decision between these alternatives

This postulated reutilization, if real, must include reutilization of large fragments of nucleic acids or nucleoproteins, as has been discussed previously[16, 17] The unusually protracted retention of radioactivity in lymphocyte RNA, as well as in DNA, suggests that there is reutilization of lymphocytes

Other kinds of data speak in favor of such a reutilization The phagocytosis of lymphocytes by reticulum cells in lymph nodes and the differentiation of these reticulum cells into new lymphocytes have been observed repeatedly[8, 18, 25] Trowell[37] recently described cells intermediate between reticulum cells and large lymphocytes that still contain pyknotic remains of phagocytosed small lymphocytes Others have made similar observations with

SOME PROBLEMS OF LYMPHOCYTE PRODUCTION*

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Though lymphocyte production has been studied for many years, it still presents a number of unsolved problems One of the first, perhaps, is to define what a lymphocyte really is Having done this, we should like to know (1) where lymphocytes are produced, and (2) in what numbers. Furthermore, the questions of the role of the lymphocyte and its ultimate destination also become involved in the study of lymphocyte production.

What Is a Lymphocyte?

For many years the lymphocyte has been studied with the ordinary microscope, both in fixed preparations and in the living state. In addition, in recent years it has been studied by phase-contrast and electron microscopy, and its absorption of ultraviolet light has also been the subject of attention A full discussion of the structure and properties of the lymphocyte may be found elsewhere (Yoffey and Courtice, 1956), in this paper we shall only touch briefly upon some of the features that have been attracting our attention in the last few years

The gradation of sizes among the small, the medium, and the large lymphocytes makes a rigorous separation of the 3 groups very difficult Size measurements are most readily made in fixed smears, and here, as is well known, the size depends upon the speed with which the smear is made as well as upon the fluid in which the cells are suspended Thus, for many years it has been our practice to prepare smears of thoracic-duct lymph only after resuspending it in serum, preferably autogenous or homogenous It is generally stated that the majority of lymphocytes in thoracic-duct lymph are small (see Reinhardt and Yoffey, 1957) Although we have not made accurate measurements, we frequently have the impression, when examining smears of thoracic-duct lymph and comparing the lymphocytes with red blood cells, that they are in fact rather medium in size than small

Whether medium or small, however, a very distinctive feature is the high ratio of nucleus to cytoplasm (N.C), so that the cytoplasm may form only a small tuft at one pole of the cell This is especially marked in the thymus and the bone marrow, and in fact we now describe this type of cell as the "polar" lymphocyte In the marrow this polarity is a particularly important morphologic criterion that is frequently maintained even when the cell has enlarged considerably and passed through a stage that has been interpreted (Yoffey, 1957) as intermediate between the small lymphocyte and the blast cell

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see Conway, 1937) and at the most active phase of the cycle there is great mitotic activity in the germinal centers, so that it is difficult to regard them as other than centers of lymphocytopoiesis

However, the presence of numerous mitoses does not necessarily mean that lymphocytes are leaving the lymphoid tissues; they could conceivably multiply and rapidly die *in situ* The data of Kindred (1942) gave some quantitative indication of the extent of lymphocyte destruction in lymphoid tissue in the rat, although on balance this destruction was very much less than the new formation

content of lymph draining a node It was shown that, after the injection of

this change was associated, after a short time (4 hours), with increased forma-

if any correlation could be established in *normal* animals between the structure of lymphoid tissue and the rate of lymphocyte production It was thought to be particularly desirable to work with perfectly healthy animals since, in these, the interpretation of the germinal center as a reaction center seemed far less likely than in animals that had been given injections of antigen

For the purposes of the present study we selected the male guinea pig of the Dunklin-Hartley strain weighing approximately 400 gm , since we already had accumulated, over a number of years, a good deal of quantitative information about the hemopoietic system in this animal With progessive improvements in technique, the later results are in all probability much more accurate than the earlier In the anesthetized animal, thoracic-duct lymph is derived mainly from the abdomen (for full discussion, see Yoffey and Courtice, 1956) From the work of Mann and Higgins (1950) it appears that this holds good even in the unanesthetized animal, at least in the rat Consequently, the lymphoid tissues from which thoracic-duct lymphocytes would be likely to arise are (1) the Peyer's patches and solitary nodules in the intestine, and (2) the glandula mesenterica magna, the large lymphoid mass at the root of the mesentery

Accordingly, in a series of 15 male guinea pigs of the Dunklin-Hartley strain, weighing about 400 gm , the thoracic-duct lymph was collected by the technique already described (Reinhardt and Yoffey, 1957), and its lymphocyte content estimated over a period of 2 hours The animals were then sacrificed, and the abdominal lymphoid tissue was examined histologically after fixation in Zenker-formol, sectioning at 3 μ, and subsequent staining with Dominici's eosin-orange G-toluidine blue, or hematoxylin and eosin

The data on lymphocyte output are presented in TABLE 1

There is no other cell occurring diffusely throughout the body in large numbers with the high N:C ratio of the lymphocyte

Another distinctive property of the lymphocyte is its characteristic type of movement. Ehrlich and Lazarus (1898), in conformity with the earlier views of Ehrlich, were impressed by the fact that lymphocytes (1) possessed no distinctive granules, unlike those that Ehrlich (1879) had described in the granulocytes after the introduction of differential staining, and (2) were incapable of movement These investigators therefore concluded that the lymphocytes were a distinct cell group, completely unrelated to any of the other cells in the blood, bone marrow, or connective tissue. Subsequently it was shown not only that lymphocytes could move, but that they did so very characteristically, the nucleus forming a rounded head and the cytoplasm a short stumpy tail (see Lewis, 1931, 1933, De Bruyn, 1945) Rich (1936) and others have used this typical movement to differentiate lymphocytes from other cells, though De Bruyn's (1945) work has cast doubt on the validity of such a criterion

The presence of a nucleolus in lymphocytes has been disputed by many, and this has been thought to fit in with the concept that the small lymphocyte is a mature cell, whereas the presence of a nucleolus has been regarded as evidence of immaturity. Nucleoli tend to be obscured in air-dried smears, though they can often be seen surrounded by an irregular coating of (DNA) desoxyribonucleic acid (Pathak, Reinhardt, and Yoffey, 1956). In supravital preparations the nucleoli stain readily with brilliant cresyl blue and frequently appear to be perfectly spherical Stockinger and Kellner (1952) obtained a similar appearance in fixed preparations, staining with methylene blue at pH 4 9. Pulvertaft and Jayne (1953), as well as Ackerman and Bellios (1955) have observed nucleoli in living lymphocytes by means of phase-contrast microscopy Nucleoli have also been seen in lymphocytes examined with the electron microscope (Bernard *et al.* 1955, Pease, 1956)

Where Are Lymphocytes Formed?

As far as we know, lymphocytes are formed in the various members of the lymphoid complex in either diffuse or nodular lymphoid tissue (the problem of

the germinal centers It is clear that germinal centers are not essential for lymphocyte formation, and that this formation can occur actively in the absence of these centers, which as a rule put in their first appearance in early postnatal life (Gyllensten, 1950, Gyllensten and Ringertz, 1954) Even in the adult there may be a considerable amount of diffuse lymphoid tissue, devoid of germinal centers, in which lymphocyte formation nevertheless is proceeding actively

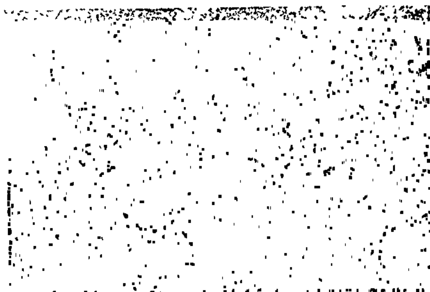


FIGURE 1 A group of nodules from experiment No 70, which had the highest lymphocyte output of the series These are not as active as the group of nodules from the same lymphnode shown in FIGURE 2 X90

flow there may have been little discharge of cells from the gland, and in fact they could accumulate in it, but subsequently, perhaps even at a time when the germinal centers were inactive, a large lymphocyte discharge might occur in association with a copious flow of lymph The most convincing experiment therefore would probably be one in which there was a sustained high output of lymphocytes over a considerable period This would allow time for stored lymphocytes to be discharged, so that in the later stages one could be sure of dealing with active lymphocyte formation

Such an experiment is No 70, in which, although there was a high lymphocyte output, the gland (FIGURES 1 to 6) contained large and active nodules, with abundant mitoses, but often very few surrounding small lymphocytes, these latter must therefore have been discharged from the gland almost as rapidly as they were formed It is difficult to see how appearances such as those found in this experiment could be explained on any basis other than that active formation of new lymphocytes was in progress

One gets the impression that these bulging, rounded, active centers may develop considerable tension, and this view is further strenghened by the compression of the surrounding framework of reticular fibers (see Conway, 1937, Plate 3, and compare Rohlich, 1928) It is of interest that it was this type of center that Heiberg (1933) interpreted as incapable of cytopoiesis, since the surrounding rim of small lymphocytes was missing From an experiment such as that just cited it is clear that it is precisely such a center that may be most

TABLE 1

THORACIC DUCT LYMPHOCYTE OUTPUT IN 15 HEALTHY GUINEA PIGS OF THE DUNKLIN-HARTLEY STRAIN

		First hour		Second hour	
No	Weight gm	Vol /hour	Cells/hour (×10⁶)	Vol /hour	Cells/hour (×10⁶)
53	456	0 94(−12%)	12 50	1 10(−13%)	9 00
55	450	1 14(+7%)	33 10	1 00(−20%)	21.20
57	440	0 94(−12%)	15 20	0 83(−34%)	13 30
58	420	0 78(−27%)	11 00	1 59(+26%)	30 70
61	397	1 11(+4%)	11 50	1 13(−10%)	7 30
63	420	0 70(−35%)	5 03	0 54(−57%)	1 99
69	419	1 46(+36%)	17 10	1 65(+31%)	18 70
70	470	0 98(−8%)	18 80	1 50(+19%)	29 80
78	405	0 75(−30%)	5 40	0 96(−24%)	2 05
79	410	1 29(+21%)	16 40	1 38(+10%)	13 60
81	380	1 26(+18%)	12 90	2 03(+61%)	15 00
89	410	1 21(+13%)	16 20	1 55(+23%)	22 60
107	394	1 06(−1%)	10 60	0 84(−34%)	7 48
110	403	1 71(+60%)	18 50	1 75(+39%)	17 90
113	372	0 77(−28%)	16 50	1 09(−13%)	33 30
Average	416	1 07 (S E * = 07)	14 4 (S E = 1 7)	1 26 (S E = 11)	15 3 (S E. = 2 7)

* S E = standard error of the mean

Correlation Between Histological Appearance and Lymphocyte Output

Some obvious difficulties of interpretation arise at the outset Thoracic-duct lymph would contain only indirect-entry lymphocytes, that is, those that reach the blood stream indirectly after first entering the lymph stream Direct-entry lymphocytes (those that find their way to the blood stream directly by passing through the walls of blood capillaries in lymphoid tissue without entering the lymph stream) could be numerous, but no indication of this would be obtained from an examination of thoracic-duct lymph Such examination can throw light only on the indirect-entry lymphocytes

We do not know how to decide, from histological examination of lymphoid tissue, the relative proportions of direct- and indirect-entry lymphocytes that it is producing, although the data on DNA turnover that we report later in this paper suggest that the direct-entry cells may considerably exceed the indirect-entry ones

However, even in relation to indirect-entry lymphocytes, obvious difficulties [illegible]

as we believe, the view of [illegible] (1937), is correct, and germinal centers undergo cyclic changes in activity, it is possible that during a period of active lymphocyte formation with poor lymph

FIGURE 4 A portion of the right margin of the nodule in FIGURE 3 Note the mitoses There are a few small lymphocytes adjoining the lymph sinus ×840

FIGURE 5 The edge of an active germinal center Again note the small lymphocytes immediately adjoining the sinus The cells at greater distances from the sinus are mostly medium lymphocytes In active nodules such as this, small lymphocytes appear to leave the nodule rapidly ×840



[illegible]
at the inner pole of the second nodule from the left ×90

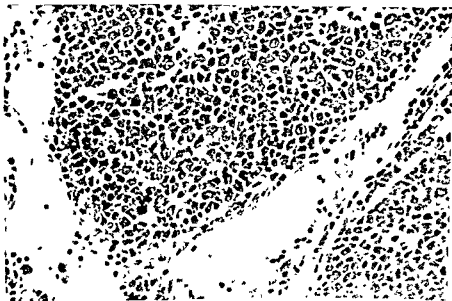


FIGURE 3 A higher magnification of the inner part of the second nodule from the left in FIGURE 2 Numerous mitoses are evident Most of the cells are medium and large lymphocytes ×400



FIGURE 7 From experiment No 53, which had a low lymphocyte output Note the numerous lymph sinuses, the lumens of which are filled with small lymphocytes. ×35

[illegible]

true that the centers all appear to be tense and the cells rather tightly packed. FIGURES 7–10, on the other hand, are from experiment No 53 in which there was a lower cell output The contrasting appearance of the germinal centers between this and the previous experiment is most striking The centers here [illegible] and large lymphocytes

Lymph sinuses filled with lymphocytes may be seen in glands from animals with either high or low lymphocyte output, but they tend to be more numerous in cases where the output is low and the flow of lymph less than normal In such cases the lymphocytes could stagnate and accumulate in the intraglandular sinuses, forming a lymphocyte store that would be quickly mobilized if the flow of lymph were increased (FIGURES 7 and 8)

We interpret FIGURES 9 and 10 as representing a stage of which active lymphocytopoiesis in the germinal center has come to an end. The center appears loose and but scantily filled, with large intercellular spaces. There are but few lymphocytes in the centers, and hardly any mitoses The surrounding rim of small lymphocytes is neither thick nor closely packed

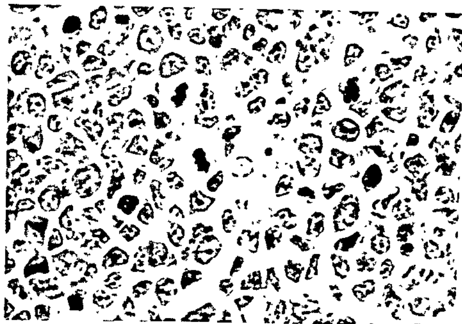


FIGURE 6 A portion of an active germinal center A macrophage with an adjacent medium lymphocyte in mitoses The macrophage is sharply demarcated from the surrounding lymphocytes ×840

active in lymphocyte formation, but this may be obscured if the newly formed lymphocytes are removed immediately from the periphery of the germinal center (FIGURES 3, 4, and 5). Active nodules such as these are frequently surrounded by dilated and almost empty lymph sinuses Presumably, as soon as small lymphocytes enter the sinuses they must be washed away by the current of lymph

In the guinea pig the lymph channels surrounding the nodules are usually devoid of a sinus reticulum such as is found so conspicuously in the dog and other mammals, and this makes it very easy to observe their lymphocyte content It is not clea
germinal center find
lymph sinuses In
be able to migrate Furthermore, in many centers there is often a zone of active mitosis in the peripheral part of the center, close to the sinuses Speculations of this nature do not take into account the occurrence of direct entry This is difficult to observe histologically, for it means fixing lymphocytes in the act of passing through the endothelium of the blood capillary Even if they were passing through at the time of fixation, the shrinkage and distortion of the fixation might well complete the process

It is in closely packed areas of large and medium lymphocytes that the most numerous mitoses are to be found (FIGURES 3–6), and in such areas there are often few or no macrophages present (FIGURE 6) Even when a few macro-

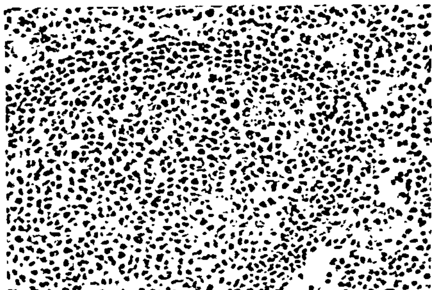


FIGURE 10. A part of the same center as that shown in FIGURE 9, but at higher magnification. Compare with the active center shown at the same magnification in FIGURE 3, in which there are many tightly packed medium and large lymphocytes. ×400

In summary, one may say that it is usually, though not invariably, possible to establish a fair degree of correlation between the histological appearance of the glandula mesenterica magna and the lymphocytes in thoracic-duct lymph. The broad correlation between the lymphoid nodules and lymphocyte output in the normal animal is in accord with the view that the germinal centers are definitely associated with lymphocyte production, whatever other role they may or may not have in the reaction of the body to noxious substances.

While it is true that lymphocyte formation can take place in diffuse lymphoid tissue, even in the absence of germinal centers, it nevertheless seems to be necessary for these centers to be present if peak levels of lymphocyte production are to be attained. They would thus seem to constitute a mechanism for boosting lymphocytopoiesis to heights that it would otherwise not attain.

It is of course also possible that there may be qualitative differences between lymphocytes produced in germinal centers and those formed elsewhere. The changes occurring in lymph nodes in response to antigenic stimulation afford ample scope for speculation along these lines. However, the data here presented have no bearing on this aspect of the problem.

Peyer's patches. Although we have not attempted to make quantitative estimations, the lymphoid tissue in the Peyer's patches appeared to be at least as active as that in the mesenteric lymph nodes, and occasionally more so (FIGURES 11 and 12). The germinal centers tend to be larger in the Peyer's patches than in the lymph node, while the mitoses are especially numerous in

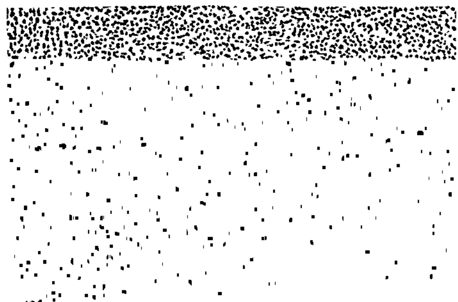


FIGURE 9 A nodule from FIGURE 7 Note the loose texture of the germinal center ×200

the peripheral part of the center, as is not infrequently the case in lymphoid tissue.

Cyclic changes and variation in lymphocyte production It has been evident for some time that lymphocyte output, at any rate as measured by thoracic-duct lymphocytes, is subject to great variation (Yoffey, 1936). This could be explained most readily on the assumption that lymphocytopoiesis occurs in cycles, the great variation that is to be found in the histological appearances of lymphoid tissue fits in admirably with such an interpretation.

Degenerative Changes in Active Lymphoid Tissue and Their Significance

A problem that always arises when one studies the histology of lymphoid

inclusions, described by Flemming in 1885 as the "tingible *Korper*" have been generally interpreted as the remains of dead lymphocytes that have been ingested by the macrophages.

There is no doubt that these macrophages can be well marked in actively growing tissue (see Kindred, 1942), and they have been interpreted as convincing evidence that the so-called germinal centers are in fact regions where lymphocytes are dying in large numbers (Ehrich, 1946, Dougherty and White, 1945) However, the later experiments of Ehrich *et al.* (1949) in which the appearance of enlarged germinal centers is associated with increased outflow of lymphocytes from the node argue strongly against this In the present observations, too, appearances such as those in FIGURES 1 to 6, indicating particu-

telius (1951 and 1957) on the biphasic appearance of radioactive lymphocytes in the blood after the administration of P^{32} Hamilton (1957) further suggested that lymphocytes might thus serve as carriers of specific nucleic acid templates, especially those concerned with antibody formation, and in this way one could explain the capacity of the organism to preserve its powers of antibody formation over many years It should perhaps be noted in this connection that our knowledge of prolonged immunity is still beset with numerous uncertainties, and other views have been advanced

Thus, Burnet and Fenner (1953) inclined to the view that the production of antibody was initiated by the entry of antigen into phagocytic cells (macrophages), and that from these cells modified antigen was then transferred to "reticulum" cells or other relatively undifferentiated mesenchymal cells in the



FIGURE 11 A part of a Peyer's patch from experiment No 70, which had a very high lymphocyte output Judging from the histological appearance, the lymphocytopoeisis in the Peyer's patches is at least as high as it is in the lymph nodes ×90

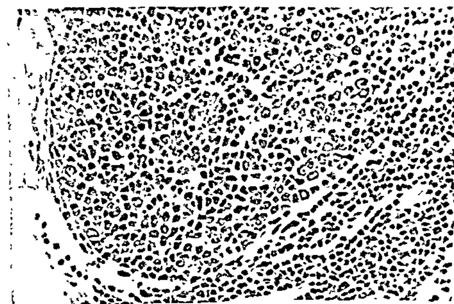


FIGURE 12 A field from FIGURE 11 at a higher magnification ×400

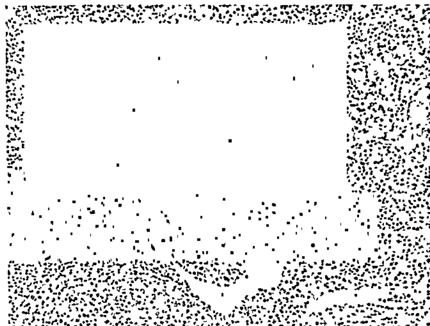


FIGURE 13 A germinal center from the most active lymph node seen thus far, from a guinea pig that had received injections of horse serum on alternate days for a fortnight ×150

cells, and with no indication of transformation into them (FIGURES 6, 13, and 14)

Our most interesting material in this connection is illustrated in FIGURES 13 and 14, taken not from a normal guinea pig, but from one that had been given repeated intraperitoneal injections of horse serum every other day for a fortnight The glandula mesenterica magna of this animal contains the most active germinal centers we have seen They are tightly packed with cells, and contain numerous mitoses. Macrophages are large and conspicuous, containing several of Flemming's stainable bodies These may or may not be the remains of lymphocytes, but it is by no means obvious that they are

In general, the tightly packed lymphocytes of the active germinal centers show hardly any sign of cell death It will be noted further that here, too, in this very active center, the zone of surrounding small lymphocytes is scarcely in evidence (FIGURES 13 and 14) It seems clear that in the most active centers the small lymphocytes do not accumulate either in or around the center, but rapidly leave to enter lymph sinuses or blood vessels. Again one may note that the macrophages in these active centers stand out very distinctly, sharply demarcated from the lymphoid cells, with no indication whatever that they may be transformed into them

immediate vicinity of the macrophages Under conditions inducing active antibody formation a varying number of these cells become active in protein synthesis and take on the staining qualities characteristic of plasma cells There could possibly be genetic mutations in individual stem cells of the antibody-forming series, giving rise to specific cell strains that might persist in the organism for many years and perhaps throughout life. Alternatively, one may

One further point should be noted in connection with the reutilization hypothesis. If the dying lymphocytes are the bearers of nucleic acid templates needed for the synthesis of new antibody protein, one might expect them to be most abundant and conspicuous in regions of active antibody formation However, as has been elegantly shown by Coons and his collaborators (Coons, Leduc, and Kaplan, 1951; Coons, Leduc, and Connolly, 1955; Leduc, Coons, and Connolly, 1955; White, Coons, and Connolly, 1955), by means of the fluorescent antibody technique, the chief site of antibody formation in lymph nodes is in the medullary cords, whereas the cortical nodules, in which the reutilization of dead lymphocytes is thought to occur, are hardly involved in the process

It should also be emphasized that the presence of dead cells in areas of active cell proliferation is by no means a phenomenon specific to the germinal centers of lymphoid tissue As De Bruyn (1948) points out, "mitotic activity is frequently accompanied with necrobiotic phenomena in developmental processes." He cites several observations to this effect, including the review by Ernst (1926), who "made a study of cellular degeneration and cell death during development and found it of general occurrence in a great variety of tissues and organs throughout the vertebrates" The latter investigator inclined to the view that dividing cells might generate a mitotic pressure that would damage cells in their vicinity While De Bruyn (1948) thinks such a mechanism unlikely, one undoubtedly frequently has the impression of tension developing in germinal centers, and the disposition of the surrounding reticulum fibers, as noted by Conway (1937), seems to be explicable most readily on the basis of their compression by a rapidly enlarging germinal center in a phase of active growth It is pertinent in this connection to refer again to the studies of Kindred (1942) who, with quantitative methods, found on occasion a surprisingly high number of dead cells in the germinal centers

The hypothesis of lymphocyte reutilization sounds quite plausible However, as Trowell (1957) emphasizes, no quantitative correlation has been established between the number of macrophages present in germinal centers and lymphocyte formation. In this situation macrophages are very variable in their distribution On the reutilization hypothesis, the more active the ger-

and 4) Furthermore, even when one sees these macrophages in active germinal centers, they stand out distinctly, sharply demarcated from the lymphoid

Sjovall (1936), who first clearly formulated the concept of a circulation of lymphocytes, thought that they absorbed toxins (both endogenous and exogenous) while in the tissues, and then returned them to the lymphoid tissues where these toxins would be neutralized Lymphocytes freed of toxins could then re-enter the blood to start the cycle of toxin-fixation once again.

Sjovall's view is but one of many variations on a theme, first developed by Virchow (1860), that lymphoid tissue is essentially defensive in its function The history of this idea, and the way in which it has repeatedly assumed new forms as older ones were shown to be untenable, is a fascinating instance of the tenacity with which ideas persist once they have taken hold. The literature in relation to bacterial invasion and the production of antibodies has already been reviewed elsewhere (Yoffey and Courtice, 1956). In recent years the lymphocyte has also been assigned a role in relation to the immunity to tissue grafts (Medawar, 1945; Scothorne and McGregor, 1955, Algire *et al.*, 1957, Darcy, 1949) Weaver (1957) has described how target cells, in porous chambers to which lymphocytes have access, seem to die after the lymphocytes have been in contact with them This would suggest the lymphocyte as a mobile and the plasma cell as a stationary source of antibody. Recently Hamilton (1957) and Trowell (1957) have given a new twist to the idea, as has already been noted, and instead of recirculation of lymphocytes to fix toxins, they advanced the concept of continuous reutilization to subserve antibody formation

This is a somewhat different concept from that of Carrel (1924), who sug- [illegible] tems "As purines have been shown to be powerful stimuli of phosphorylation, or more precisely, since they act as phosphorus-transferring enzymes, our results may be interpreted to mean that lymphocytolysis furnishes not only building stones, but also energy for synthetic processes "

When Sjovall (1936) first put forward the idea of a lymphocyte circulation he compared the lymph vessels and lymphoid tissues to a particle-containing lake and argued that if at any time there were more particles in the water leaving the lake than entering it, this did not prove that the particles were necessarily formed in the lake, but merely that they were present in it when the [illegible]

concluded that there was a slight recirculation of lymphocytes in the cat and dog Their data suggested that, for every 30 lymphocytes entering the blood stream from the lymph, 1 would find its way back to the lymph stream To the extent that the total lymphocyte output is greater than the lymphocytes found in the thoracic duct, the ratio of circulating to noncirculating lymphocytes could be proportionately even less The findings of Goodall and Paton (1905–1906) and of Baker (1932–1933) indicate that in the cat and dog a similar state of affairs holds good for intestinal lymph, the main source of thoracic-



[illegible] Note the [illegible] or the most [illegible] and corner, [illegible]

Reutilization on any appreciable scale would also appear to render unnecessary the extensive synthesis of new DNA In fact, however, lymphoid tissue shows continuous and rapid DNA synthesis, as measured by the incorporation of P^{32}. This has been demonstrated by chemical estimation, as in the earlier work of Andreasen and Ottesen (1945) and in our own data It has also been shown very clearly in radioautographs, such as those of Gyllensten and Rin- [illegible] intraperitoneal [illegible] the cells of the [illegible] ne phenomenon from another approach; namely, the passage of newly formed lymphocytes into the blood (see also Hamilton, 1957, Osgood *et al*, 1952, Ottesen 1954) The concept of lymphocyte reutilization must therefore be regarded as a speculation that is intriguing, but far from proved

The Problem of Lymphocyte Circulation

At various times the view has been advanced that the production of lymphocytes in large numbers is more apparent than real and that, in fact, the majority of the lymphocytes are continuously recirculating between the blood stream, on the one hand, and the lymph vessels and lymphoid tissues on the other

without any apparent effect either on the general condition of the patients or the composition of the blood, whereas in the case observed by Courtice *et al.* the fistula was associated with impairment of the general condition and lowering of the plasma proteins

From the point of view of the hypothesis of a lymphocyte circulation, the most interesting experiments have been those performed on animals. Mann and Higgins (1950) maintained a thoracic-duct fistula in several instances for 6 days, Shrewsbury and Reinhardt (1952) for 4 days, and Glenn *et al.* (1949) for periods up to 12 days. More recently Gowans (1957) maintained fistulae for 4 days In all these experiments the thoracic-duct lymphocyte output showed a steady decrease, while at the same time there was a fall in the blood lymphocytes At first sight, results of this type seem to fit in very well with the concept of a lymphocyte circulation, but fuller analysis of the ways in which lymphocytes can reach the blood indicate that the situation is somewhat more complex than is at first evident.

Channels Through Which Lymphocytes Enter the Blood

The problem of lymphocyte production was for many years regarded as synonymous with thoracic-duct lymphocyte output, but an increasing body of evidence is accumulating to indicate that this is not the case There appear to be 4 major paths by which lymphocytes may reach the blood after their formation These are (1) the thoracic duct, (2) the right lymph duct (3) other and probably smaller lymphaticovenous communications in the thorax and abdomen, and (4) direct entry into the blood stream through the walls of the blood capillaries in lymphoid tissue We may refer to paths 1–3 as indirect-entry lymphocytes, since the lymphocytes reach the blood only indirectly and must first enter the lymph stream, in contradistinction to those reaching the blood by path 4, which are direct-entry lymphocytes

Most experimental attempts to assess lymphocyte production have concentrated on the indirect-entry (lymph-borne) lymphocytes The main lymph ducts provide a convenient bottleneck, where the lymph from the greater part of the body can be collected and its lymphocyte content estimated Unfortunately, the only duct that has been readily accessible is the thoracic duct Few direct observations have been made on the right lymph duct, while the remaining lymphaticovenous anastomoses, because of their irregularity in size and distribution, are completely unknown quantities in this respect. Evidently, thoracic-duct lymphocytes, if they are newly formed, are only a part of the total lymphocyte output (Yoffey, 1936) However, how big a part?

Information about the number of lymphocytes reaching the blood via the right lymph duct is scanty Some observations made by Warren and Drinker (1942) in the dog suggest that at times the number may be quite appreciable Since the cannulation of the right lymph duct presents considerably greater technical difficulty than does that of the thoracic duct, information concerning it has also been derived from experiments in which the thoracic duct has been cannulated after preliminary ligation of the right duct and allowing time for the enlargement of anastomoses between it and the thoracic duct Hughes *et al* (1956) found that in an experiment of this kind in the rabbit the cell

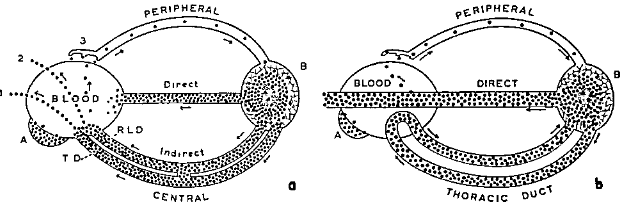
duct lymph in the anesthetized animal. Allen (1945) obtained results of the same order with diaphragmatic lymph in rabbits, but also thought that the tissue fluid, exemplified in this instance by the peritoneal fluid (Allen, 1936; Maurer *et al* 1940), contained 4 to 5 times as many cells as peripheral lymph Nii (1932) in the leg lymph of 10 rabbits found a mean count of 2230 cells per cu. mm., of which 4.14 per cent were erythrocytes, the range being 1100 to 3550 Okaue and Hojo (1935–1936) found, in 15 rabbits, counts of the same order in leg lymph, but a considerably higher count in testicular lymph, the mean on one side being 2853 cells per cu. mm. and on the other 3541 per cu. mm , about 90 per cent of the cells being lymphocytes It is possible that there may be more cells entering peripheral lymph in smaller animals than in larger ones, and this may contribute to the fact that the thoracic-duct lymphocyte output tends to be higher in the smaller (Yoffey and Courtice, 1956, their Figure 83, page 343) If lymphocyte production is also increased, then the ratio between cells in peripheral and central lymph need not be markedly affected.

In view of the relatively low lymphocyte concentration in peripheral as compared with central lymph, one must assume that if an extensive circulation of lymphocytes occurs, its most likely site would be in the actual substance of the lymphoid tissues themselves, as suggested, for example, by Farr (1951) and by Fichtelius (1953) However, it is very difficult to see how the structure of the lymph node would accord with the concept of lymphocyte circulation on an extensive scale.

In terms of the structure of the lymph glands, the lymph sinuses are the obvious counterpart of Sjovall's lake, and it is true that on occasion (FIGURES 7 and 8) one sees in intraglandular vessels accumulations of lymphocytes that would accord with the analogy of a lake However, this appearance is infrequent, and is presumably due to a temporary stagnation of lymph When an active current is resumed, the "lake" would rapidly clear Apart from the lymph channels, the diffuse lymphoid tissue and the germinal centers appear to be areas of active cell growth, not of cell storage The evidence of

the work of Gyllensten and Ringertz (1954), and Gyllensten *et al* (1956) showing by radioautographic methods the rapid uptake of DNAP[32] in germinal centers, seems difficult to explain on any other basis than that of active cell multiplication, and this is further substantiated by the data on DNA synthesis of Andreasen and Ottesen (1945), Fichtelius (1953), and those that we present later in this paper

In recent years the concept of a lymphocyte circulation has received its main support from experiments based on prolonged drainage of lymph from the thoracic duct Such prolonged drainage has been made possible by the [illegible] (Mann and Higgins, 1950; Shrewsbury and Reinhardt, [illegible] ats, Glenn *et al* , 1949, with dogs, Courtice [illegible] *l* , 1953, in man) In the experiments of [illegible] was draining quite freely for 2 to 11 days



FIGURES 15*a* and *b* These two diagrams represent schematically two different ways of interpreting some of the known facts concerning the life cycle of the lymphocyte The lymphocytes are formed in the lymphoid tissue, and they may enter the blood in either of 2 ways (1) *indirectly*, after entering the lymph stream through the thoracic duct, the right lymph duct, and the other lymphaticovenous communications not shown in the diagrams (anastomoses are usually present between the thoracic duct and the right lymph duct) and, (2) *directly*, by passing through the walls of the blood capillaries within the lymphoid tissues Data on DNA turnover, in addition to earlier mitotic studies, suggest that direct-entry lymphocytes may be even more numerous than indirect entry ones Most of the lymphocytes entering the blood are newly formed. Some observers believe that the indirect entry lymphocytes consist mainly of cells continually circulating between the blood and the lymph

FIGURE 15*a* is based on the view of Yoffey and Drinker (1939) that there is only a minor recirculation of lymphocytes Both direct- and indirect-entry lymphocytes were thought to consist, in the cat and the dog, of newly formed cells

FIGURE 15*b* allows for the extensive recirculation of indirect-entry lymphocytes, and this recirculation is shown as taking place via the thoracic duct, since the experimental work suggesting recirculation has been based on studies of thoracic-duct lymph If the recirculation hypothesis is correct, the same thing would presumably hold true for other indirect-entry lymphocytes Since mitotic and DNA turnover studies indicate continuous new formation of lymphocytes on a large scale, one must assume, if the majority of the indirect-entry lymphocytes are recirculating, that the bulk of the newly formed lymphocytes are to be found in the direct entry group

A = spleen, B = lymph nodes and other lymphoid tissue, R L D = right lymph duct, T D = thoracic duct

output of the thoracic duct was approximately doubled. As against this, Glenn *et al.* (1949) found in the dog that ligating the right lymph duct made very little difference to the cell content of lymph from the thoracic duct. Although these results are not therefore conclusive, they do suggest that one must seriously reckon, at any rate in some animals, with the possibility that large numbers of lymphocytes occasionally may enter the blood via the right lymph duct.

This is not a very precise conclusion, but the position is even more obscure with the 2 remaining groups of lymphocytes: those reaching the blood through lymphaticovenous channels other than the thoracic or right lymph ducts and those entering directly. The obvious way of overcoming all these difficulties is by a study of lymphocyte formation at its source, in the lymphoid tissue itself, and this has been d

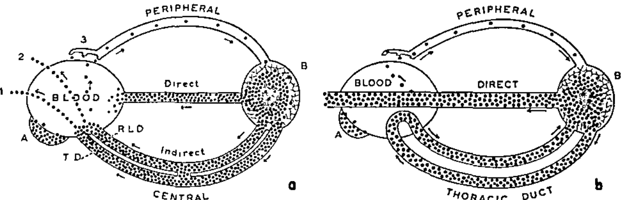
(1942) and of Andreasen and

over, as has been done by

Ringertz (1954), and as reported in this paper. Kindred (1942) made his calculations on the basis of both 1-hour and 30-minute mitotic cycles. In the former case he estimated there was a total lymphocyte production of 2.8×10^6 per 100 gm. per hour and, in the latter case, 6.9×10^6 per 100 gm. per hour. This compares with a figure of 1.1×10^6 derived from counts of thoracic-duct lymph (Hungerford, Reinhardt, and Li, 1952). Working with Kindred's data, Leblond and Stevens (1948) calculated that the lymphocytes in lymphoid tissue must have a mean doubling time of about 48 hours. Andreasen and Christensen (1949), who also studied mitotic activity in the lymphoid organs of the rat, worked with isolated nuclei and used the citric acid technique to obtain their nuclear suspension

The alternative to a study of mitoses in sections is the determination of DNA turnover The evidence derived from turnover studies suggests that, including the thymus and the intestinal lymphoid tissue, the total output of lymphocytes in the guinea pig may be about 2.5 times as great as the thoracic-duct output. Even if it should therefore transpire that a large number of the cells in thoracic-duct lymph are recirculating and not newly formed, and even if this holds true in the case of the other indirect-entry lymphocytes, the data on DNA turnover nevertheless indicate the massive formation of new lymphocytes on a large scale, and one would then have to postulate that these enter the blood directly. There would be a daily replacement factor (DRF)

indirect-entry lymphocytes are recirculating However, in either case we must assume, on the basis of the DNA turnover figures, that the direct-entry lymphocytes are considerably more numerous than the indirect-entry ones In the case of the guinea pig the direct-entry lymphocytes would appear to be approximately 2 to 3 times as numerous as those reaching the blood via the thoracic duct The D I ratio (that is, the ratio of direct-entry to indirect-entry lymphocytes) presumably could be even higher, and it may be, in cases



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A = spleen, B = lymph nodes and other lymphoid tissue, R L D = right lymph duct, T D = thoracic duct

such as these, that one finds a lymphocytosis developing during the course of an experiment in which the thoracic duct is cannulated and its lymph diverted to the exterior (Sanders *et al*, 1940). A high rate of direct entry makes it impossible to calculate the index of lymphocyte replacement on the basis of the thoracic-duct lymphocyte output, as was attempted in earlier work (cf. Yoffey and Courtice, 1956). Furthermore, it renders much more difficult *the quantitative study of lymphocytosis and lymphopenia, although the* more limited knowledge of thoracic-duct lymphocyte output might still be of some value as an indication of trends in lymphocyte production, if it could be established that the D:I ratio is approximately constant.

Assessment of Lymphocyte Production by Means of DNA Turnover

In view of the difficulties of assessing lymphocyte production by studying the cell content of thoracic-duct lymph, or even those of the thoracic duct and the right lymph duct combined, it was decided to investigate lymphocyte pro-

into DNA only when it is being newly synthesized. The incorporation occurs during interphase in preparation for mitosis, and therefore the rate of DNA synthesis as measured by isotope incorporation is a direct measure of the rate of mitosis, that is, of new cell formation (Leblond and Walker, 1956; Thomson *et al.*, 1953)

Some excellent work on DNA turnover in lymphoid tissues has already been done by Andreasen and Ottesen (1944, 1945) in rats In their first studies (1944) labeled phosphate was administered subcutaneously and was estimated in the DNA of lymphoid organs 2 hours and 42 hours later The activity of DNAP^{32} was then compared with that of plasma phosphate. The data of these investigators showed that if over a period the newly formed nucleic acid in the thymus = 100, then intestinal lymph nodes = 15 6, lymph nodes of lungs and skin = 17 5, spleen = 16 3, and Peyer's patches = 10 5 Thus *they found DNA turnover much greater in the thymus than in other lymphoid* tissues Their 1945 study confirmed this "A large nucleic acid turnover is observed in the lymphoid organs The greatest renewal takes place in the thymus, the rate of renewal in the mature animals being 5–6% during the 3 hours' experimental period The corresponding figures for the lymph nodes and spleen are only 1–2% "

sive review by Leblond and Walker, 1956) the general principle became established that all cells, with some minor exceptions, contain the same amount of DNA, and it therefore became possible to calculate cell production from DNA synthesis. In the present paper there are presented the preliminary results of an attempt to assess in this manner the production of cells in lymphoid tissue. However, it should be emphasized that the results are only an approximation, and later may need revision.

Experimental details. For the determination of the rate of DNA renewal 16 male guinea pigs (mean weight, 412 gm) were injected intraperitoneally with 90 μc. of $Na_2HP^{32}O_4$. One half of the animals were sacrificed at 2 hours and the remainder at 5 hours. In dissecting out the lymphoid tissue an attempt was made to include as many of the macroscopically visible nodes as possible. Mean weight was 0 38 per cent of body weight. The group entitled "cervical" included the nodes from the neck region and also the axillary and inguinal nodes. The group entitled "mesenteric" included the nodes at the root of the mesentery, the renal, hepatic, and lumbar nodes, and also the mediastinal nodes. Thymus and intestinal lymphoid tissue have not been included. The specific activity of the acid-soluble phosphate and DNA phosphate were determined after isolation by a modified Schmidt-Thannhauser technique. The cell numbers in the various groups were determined in 4 animals by analyzing for total DNA content by the method of Schneider (1945) and dividing by the average amount of DNA per cell obtained by the same procedure on a suspension containing a known number of cells

The percentage renewal per hour for DNA was calculated by the following formula·

$$\frac{(\text{DNA 5-hour S A.}^* - \text{DNA 2-hour S.A}) \times 100}{\text{Average S A of acid-soluble phosphate 3 hours}}$$

A summary of the results is given in TABLE 2, and a number of comments must be made The splenic contribution is unexpectedly high. If one compares our results with Kindred's (1942) estimates in the rat, his figure of 67 6 × 10⁶ lymphocytes per 100 gm per day on a 1-hour mitotic cycle, or 166 × 10⁶ on a half-hour cycle, is still well below the present figure based on DNA turnover, namely (in a 400-gm guinea pig) 1320 × 10⁶, if one includes the splenic component, 984 × 10⁶ if one excludes it As far as the Bristol figures are concerned, the thoracic-duct output per day (TABLE 1) = 358 × 10⁶ on a purely inbred strain, the Dunklin-Hartley (mean weight 416 gm.). The California figures (TABLE 2), based on DNA turnover, were obtained with a mixed colony of unknown strains and of approximately the same mean weight as the Bristol series (TABLE 1).

The splenic output of new cells, as just noted, is surprisingly large It may be that in part this is due to a limited production of erythrocytes, not of lymphocytes The precise estimation of splenic lymphocytopoiesis has always been a source of difficulty (Yoffey and Courtice, 1956), and the presence of foci of erythropoiesis and granulopoiesis in the smaller laboratory animals introduces an additional complication when attempting to estimate it on the basis of the DNA turnover However, in the guinea pig this splenic hemopoiesis does not seem to be as intense as in the rat or mouse

The daily formation of new lymphocytes as measured by the DNA turnover in the "mesenteric" nodes is 600 × 10⁶ This compares with a thoracic-duct output (TABLE 1) of 358 × 10⁶, which would not be draining all the mediastinal nodes This correspondence between the 2 methods of estimating

* S A specific activities These are calculated as counts per minute (cpm) per mg DNA phosphorus × 100, divided by cpm injected per gram

TABLE 2
LYMPHOID TISSUE RENEWAL IN GUINEA PIGS
(Male mean weight = 412 gm)

	DNA = specific activity*		Mean acid soluble phosphate specific activity	Calculated DNA renewal	Total cell number	Calculated cell production per hour
	2 hours	5 hours				
"Mesenteric" nodes	6 2	12 0	130	1 5%/hr	17×10^8	25×10^6
"Cervical" nodes	4 0	8 0	127	1 1%/hr	15×10^8	16×10^6
Spleen	4.1	7 9	119	1 1%/hr	13×10^8	14×10^6

* Specific activities are calculated as counts per minute (cpm) per mg DNA phosphorus times 100 divided by cpm injected per gram

lymphocyte production might mean that the direct-entry lymphocytes are not so numerous after all, and also that the bulk of the thoracic-duct lymphocytes are newly formed On the other hand, it could still be argued that the thoracic-duct lymphocytes are recirculating, while the newly formed lymphocytes enter the blood directly

In order to measure exactly the rate of DNA synthesis one would have to know the specific activity of the immediate precursor. As an approximation, the data in TABLE 2 are based upon the specific activity of the acid-soluble phosphate It is exceedingly probable that the true values do not differ from this by as much as a factor of 2, but this is a major source of uncertainty in our calculations

Finally, it is to be noted that the data in TABLE 2 do not include the thymus, Peyer's patches, or solitary intestinal nodules * In view of the observations previously noted in the present paper about the high degree of lymphocyte ... examination (FIGURES ... tinal lymphoid tissue

The Lymphocyte in Bone Marrow

... pulation, a ... became available ... , 1954, Harris, *et al*, 1956; Yoffey 1955, Yoffey 1956, Yoffey 1957) With the recent data of Hudson (1958) on marrow volume, it is now possible to calculate the ... marrow For every lymphocyte *normally* ... via the thoracic duct, and 23 are in ... lymphocyte formation are based on DNA turnover studies, the daily production in the guinea pig is about 2 5 times the thoracic-duct output Therefore, as far as numbers are concerned, the lymphocytes in the marrow could readily be derived from those produced in lymphoid tissue.

* The additional lymphocytes from these sources would doubtless make a considerable addition to the total Reference has already been made to the importance of the thymus in the formation of lymphocytes

It is important, however, before discussing this problem further, to bear in mind that the identity of the marrow lymphocyte has been the subject of a great deal of controversy. Sabin *et al.* (1925) were of the opinion that there were few or no lymphocytes in normal marrow, but that the cell that was so regarded was their "primitive" cell. They maintained at first that by means of the supravital staining technique the primitive cell could readily be distinguished from the small lymphocyte. In addition to the morphologic criteria that made this distinction possible, these investigators asserted: "These primitive cells are not very numerous; they are pretty evenly scattered throughout the marrow" From a quantitative point of view, it would be difficult to apply the description "not very numerous" to the marrow cells that we have [illegible]

As far as morphologic criteria are concerned, it is of interest to note that in later publications Sabin and her co-workers became rather more dubious about the distinction between the 2 cell types. Thus, in 1936, Sabin *et al.* wrote. " this cell, though it looks much like the small lymphocyte, lacks certain signs of differentiation. The differences though meagre are worthy of consideration." By 1938 Sabin and Miller had come to the conclusion that the morphologic criteria for distinguishing primitive cells from small lymphocytes were unsatisfactory, though they concluded on other grounds that the 2 cells were distinct and that the small lymphocyte was a mature functioning cell. These grounds were (1) the experiments of Wiseman (1931) on the lymphocyte response to foreign proteins, and (2), the involvement of the lymphocyte in the response to tuberculous infection In the light of later work these grounds do no appear adequate

The problem is fundamental We think that these cells are small lymphocytes, for the following reasons

(1) They are morphologically indistinguishable from small lymphocytes, whether in supravital preparations (Schwind, 1950) or in dried smears This is in agreement with the view expressed by Sabin and Miller (1938), the transitional cell (Yoffey, 1957) was not described as such by Sabin *et al* (1925) but was implicit in their work, and it introduces an obvious source of difficulty

(2) These cells are present in the marrow in very large numbers, but there is no sign of their formation in the marrow

(3) They are frequently seen in the marrow sinusoids as well as in the marrow parenchyma, and the cells in these 2 situations are indistinguishable from one another The cells in the sinusoids must presumably be lymphocytes, no one has yet suggested that there are numerous "primitive" cells in the circulating blood We not infrequently see such surprisingly large numbers of lymphocytes in the sinusoids that we have come to employ a [illegible] for it [illegible]

(4) [illegible]

lymp [illegible] compounds E and F had no obvious effect on the marrow lymphocytes (see

also Yoffey *et al*, 1951; Hudson *et al*, 1952). However, Fruhman and Gordon (1955a, 1955b), also employing a quantitative technique, subsequently showed that in the rat both corticosterone and hydroxycorticosterone exerted a lymphopenic effect in the marrow.

However, even if it be admitted that these cells are lymphocytes, the question as to whether they are myelogenous or hematogenous is such a fundamental one that it merits the fullest consideration. The numbers involved are such that, if lymphocytes are being formed in the marrow and discharged into the blood, they could constitute a very substantial addition to the already large number of lymphocytes entering the blood from other sources. Thus, if the marrow lymphocytes had a doubling time of 24 hours, this would be equivalent to a daily replacement factor (DRF) of 20, while a doubling time of 4 days would still be equivalent to a DRF of 5, the problem of the large numbers of lymphocytes daily passing into and disappearing from the blood would thus become more perplexing than ever.

On the whole, the evidence appears clearly to favor the view that the marrow lymphocytes are hematogenous in origin and not myelogenous. The main reasons for this view are:

(1) There is no organized lymphoid tissue, either nodular or diffuse, in the marrow, such as is associated elsewhere with lymphocyte multiplication.

(2) The lymphocytes are for the greater part scattered diffusely throughout the marrow parenchyma. This is the type of distribution one would expect if there were random migration of lymphocytes into the marrow from the blood.

(3) There is no evidence of homoplastic formation of lymphocytes in the marrow (for full discussion, see Yoffey and Courtice, 1956). The existence of the "transitional" cell cannot be interpreted as evidence of heteroplastic formation of lymphocytes from blast cells, but rather as a change in the reverse direction. This, incidentally, would mean attributing to the small lymphocyte in bone marrow the role that Sabin *et al* (1925) attributed to the "primitive" cell.

(4) The marrow lymphocytes can increase rapidly in a period that is too short to cover their local multiplication (Harris *et al*, 1956), under such circumstances they must be hematogenous in origin. There is abundant clinical evidence on these lines. In conditions of maturation arrest, more especially of the myeloid cells, there can be a rapid and massive accumulation of lymphocytes in the marrow, again without any evidence of multiplication *in situ*. When normal maturation is resumed the lymphocytes disappear from the marrow with equal rapidity without any evidence of their discharge into the blood.

Transfusion experiments. If the lymphocytes that enter the blood are destined to leave it in the bone marrow, and there to become stem cells for the development of erythrocytes and granulocytes, one would expect that, fol-

being transfused should resemble as closely as possible those normally enter

the blood. It is here, in the selection of donor lymphocytes, that the first major element of doubt arises. Lymphocyte suspensions obtained by mincing lymphoid tissues, apart from the possible damage incurred during preparation, might consist in the main of cells that were not at the correct stage of development. Thoracic-duct lymphocytes, on the other hand, would appear to be free from such objections, provided they do not belong to a special recirculating group

The experiments of Campbell and Ross (1952), who used thoracic-duct lymphocytes, yielded negative results in that the transfused lymphocytes failed to give protection Apart from the points already raised there may be a number of reasons for this failure, and experiments on these lines need to be repeated with a number of modifications before they can be regarded as decisive. It may be that scrupulous care should be exercised in the manipulation of the lymphocytes in the time between their collection and subsequent transfusion Thus, Ebert *et al.* (1940) noted that lymphocytes were very susceptible to oxygen shortage, an observation borne out by the tissue culture experiments of Trowell (1955). Furthermore, the experiments of Harris (1956), who studied the changes in guinea pig bone marrow after nonlethal doses of irradiation, seem to indicate that a very large number of lymphocytes accumulate in the marrow before regeneration of the erythroid and myeloid series really gets under way.

It is possible also that normal (but not irradiated) marrow contains some specific stimulus to further lymphocyte development. Even if it is true that lymphocytes are filtered out of the blood stream into the bone marrow to act as stem cells, one must postulate that the stimulus to the final lymphocyte transformation must be localized in the marrow, or at any rate must operate only after these cells leave the lymphoid tissues, since otherwise these tissues would function regularly as sites of erythropoiesis and myelopoiesis, instead of doing so only on rare and infrequent occasions

That transfused cells from normal donors can multiply in the hemopoietic system of irradiated animals has been shown clearly by using donor cells with a special biological marking. Thus Ford *et al* (1956) transfused cells from a strain of mice that had an atypical chromosome, so that the donor cells could be identified readily in the recipient tissues Cell suspensions prepared from the spleen were readily identified in the recipient marrow Nowell *et al* (1956) injected rat marrow cells intravenously into irradiated mice and subsequently identified them readily, since rat leukocytes give a strongly positive histochemical reaction for alkaline phosphatase, whereas those of the mouse are negative Porter (1957), working with rabbits, injected marrow suspensions from a female into irradiated males and identified the donor cells by their characteristic drumstick appearance

However, it is still not clear whether some humoral factor may not also be involved Though Urso and Congdon (1957) have shown that the success of marrow transfusions is closely related to the number of cells injected, this does not altogether rule out a humoral factor Allen *et al* (1956) found that cell-free splenic plasma from normal rabbits exerts a definite protective action when given intraperitoneally to irradiated rabbits Hilfinger *et al* (1953)

also Yoffey *et al*, 1951; Hudson *et al*, 1952). However, Fruhman and Gordon (1955a, 1955b), also employing a quantitative technique, subsequently showed that in the rat both corticosterone and hydroxycorticosterone exerted a lymphopenic effect in the marrow.

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Transfusion experiments If the lymphocytes that enter the blood are destined to leave it in the bone marrow, and there to become stem cells for the development of erythrocytes and granulocytes, one would expect that, following lethal doses of irradiation, the transfusion of lymphocytes would effectively stimulate marrow regeneration and so save life In planning such experiments it would obviously seem desirable that the lymphocytes that were being transfused should resemble as closely as possible those normally entering

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found that the cell-free supernatant fluid from marrow suspensions had a marked protective action when injected into irradiated rabbits 3 days after irradiation. It may be, therefore, that 2 factors are required, cells and a humoral adjuvant; spleen and bone marrow contain both these elements (lymphoid tissue does not contain them).

In the earlier experiments of Jacobson *et al.* (1951) it was noted that shielding of the intestine or the liver afforded a high degree of protection against irradiation damage (see their Table V, p 690), and it is probable that in the process of shielding the liver they also shielded some of the abdominal lymphoid tissue. They themselves attributed the effectiveness of the intestinal shielding to "reticulo-endothelial constituents" In view of the observations *made earlier in this paper on the lymphocytopoietic activity of Peyer's patches,* it is clear that the shielding of lymphocytes may be a factor to be considered It is obviously desirable not only that lymphocyte transfusion experiments be repeated and amplified in a number of ways, but also that these shielding experiments be confirmed and extended.

That there is a close cellular kinship between the bone marrow and the lymphoid complex is indicated by the fact that transfusion of marrow cells into irradiated animals stimulates the regeneration of the lymph node and thymus (Jacobson *et al*, 1954; Congdon and Lorenz, 1954, Fishler *et al.*, 1954, Urso and Congdon 1957, Porter, 1957) The work of Urso and Congdon (1957) suggests that here, too, there is a quantitative relationship between the number of marrow cells transfused and the speed and extent of thymic regeneration Repopulation of lymph nodes and thymus by marrow suspensions presumably can be effected only by lymphocytes and thymocytes or by a common stem cell, either alternative raises difficult problems.

The experiments of Urso and Congdon (1957) bring out one further point worthy of note The depleted marrow can rapidly remove large numbers of cells from the blood It may well be that it is marrow depletion that is the primary factor in the uptake of large numbers of lymphocytes from the blood, as in the experiments of Harris *et al* (1956) The depletion in this instance was due to the discharge from the marrow of large numbers of granulocytes in the space of four hours

Should it eventually prove that lymphocytes are not filtered out of the blood by the bone marrow to function as stem cells, then the problem of the marrow cells that occur in such large numbers, which we consider lymphocytes for reasons already given, becomes even more perplexing than it is at present

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EFFECTS OF STEROIDS ON ASPECTS OF THE METABOLISM AND FUNCTIONS OF THE LYMPHOCYTE: A HYPOTHESIS OF THE CELLULAR MECHANISMS IN ANTIBODY FORMATION AND RELATED IMMUNE PHENOMENA*

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The lymphocyte has been established as an end cell of the action of certain adrenal steroids.[1, 2] The administration of cortisone or other glycogenic adrenal cortical steroids in a variety of species, including man, produces involution of lymphoid tissue, with characteristic histological alterations in lymphoid structures.[2, 3a] Acutely, injection of one of these adrenal cortical steroids will cause an absolute lymphopenia three to six hours following hormone administration.[4, 5] This lymphopenia is based on the lymphocytolytic action of the adrenal steroids on lymphocytes and the inhibition of mitosis of lymphoid cells in lymphoid organs, with consequent failure of delivery of normal numbers of lymphocytes to the circulation. The above alterations in lymphoid tissue size and histology and in the numbers of circulating lymphocytes occur when the blood levels of certain adrenal cortical steroids, for example, hydrocortisone, are elevated.[3] This may ensue either as a result of administration of these steroids or their augmented endogenous release in re- [illegible]

[illegible]

tions that lymphoid tissue involution in the absence of the adrenals or hypophysis has been described experimentally only with 3 other groups of agents, namely, high-energy radiation (for example, X rays[6, 7]), the nitrogen mustards,[8, 9] and large doses of desoxycorticosterone[10] and sex hormones.[11, 12]

[illegible]

lism,[13-17] as well as to specific aspects of protein metabolism, for example, antibody production.[1, 16, 18, 19]

The evidence suggesting a role of the lymphocyte in protein metabolism has provided a basis for further studies of the nitrogen metabolism of this cell and of possible influences of steroid hormones on this metabolism. In addition, inasmuch as the integrity of the lymphocyte is probably based in part upon

* The data reported [illegible]

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20 per cent H_2SO_4 was added to each flask to terminate reactions and release all CO_2, and the flasks were again attached to the manometers and shaken for 20 min The alkali paper was removed from the center well, placed in a flask with the washings from the center well, carrier Na_2CO_3 solution added, and the carbonate precipitated with $BaCl_2$. The $BaCO_3$ was filtered, dried, and counted for radioactivity in the Nuclear Model 192 Ultrascaler.* All counts were corrected to infinite thickness

In experiments in which thymus cell suspensions were used, washed cell suspensions were prepared essentially as described by Farber, Kit, and Greenberg[22] in their modified Krebs-Ringer phosphate buffer, pH 7 4, using essentially the steps described by Blecher and White[23] Cell counts were made on diluted aliquots of the cell suspensions, which contained approximately 1×10^9 cells per ml.

Incubations with cell suspensions were conducted in Warburg vessels con- [illegible]

suspension and buffer added as above Steroids were added in this manner, in contrast to above, in order to maintain the tonicity of the environment of the cells. Preliminary incubation and subsequent steps were conducted as described above for mesenteric lymph nodes except that room air was the gas phase

Tumor cell suspensions were prepared by a modification of the procedure used for thymus cells After mincing the tumor in the cold, it was washed through a No 14 mesh Monel metal screen with the Krebs-Ringer buffer. The suspension was then washed successively through No 24 mesh and No 60 mesh Monel metal screens by gentle agitation, no tissue debris was forced through the screens * The cells were centrifuged for 5 min at 500 rpm and washed again with buffer After recentrifugation, the cells were suspended in an equal volume of buffer

Glycine-2-C¹⁴ incorporation studies Thymic and lymphosarcoma cell suspensions were prepared as described above Each incubation vessel contained from 4 to 8×10^8 cells, 20 μmoles glucose, 4 or 8 μmoles glycine-2-C^{14}† (1 μc), and modified Krebs-Ringer phosphate buffer, pH 7 4, to make a final volume of 2 0 ml , the gas phase was air and incubation temperature 37° C In respiration studies, provisions were made to absorb atmospheric carbon dioxide When steroids were used, each was added to the appropriate vessel in alcoholic solution, with subsequent evaporation of solvent by a gentle stream of warm air Incubations were terminated by the addition of an equal volume of cold 20 per cent trichloroacetic acid, which was 0 8 M with nonradioactive glycine Precipitated material was washed with cold trichloroacetic acid to remove acid-soluble compounds, washed with organic solvents to remove lipids, then extracted with hot 5 per cent trichloroacetic acid to isolate nucleic acids Purified protein samples were dried, and then plated on filter paper circles for

* This is essentially the same procedure described by others (Kaltenbach and Kaltenbach[24])
† Produced by the New England Nuclear Corp , Boston, Mass

energy-producing reactions, experiments have been initiated to examine the *in vitro* and *in vivo* effects of various steroids on carbohydrate oxidation by normal and malignant lymphoid tissue.

The experiments described in this paper represent a portion of studies in progress on the carbohydrate and nitrogen metabolism of lymphoid tissue The data presented were obtained in studies conducted in collaboration with Lillian A Jedeikin[20] and Melvin Blecher[21]

Experimental

Animals Male rats of the Sprague-Dawley strain* weighing 140 to 200 gm have been used as the source of lymphoid tissue. The animals were sacrificed by decapitation, and the thymus, mesenteric lymph nodes, or transplantable Murphy-Sturm lymphosarcoma† were rapidly removed by dissection The lymphosarcoma was harvested 2 to 3 weeks following transplantation in the host Adrenalectomized rats were used 2 to 3 weeks postoperatively. All animals received Rockland Rat Pellets‡ and water *ad libitum*, except for the adrenalectomized rats, which were given 1 per cent NaCl solution as drinking fluid

In the *in vivo* experiments, a single subcutaneous or intraperitoneal injection of steroid,§ 5 mg /100 gm of body weight, was made 4 hours prior to sacrifice of the animal

Glucose oxidation studies The experiments with mesenteric lymph nodes were conducted with tissue obtained, for the most part, from adrenalectomized rats The nodes were separat[illegible]
imately 2 mm. in diameter a[illegible]
nodes was added to a chilled [illegible]
of 0 076 M NaCl and 9 1 × 10^{-3} M phosphate buffer, *p*H 7 8 Steroids, when used, were added in highly dispersed aqueous suspension, incubations were conducted with air as the gas phase The center well of the Warburg flask contained filter paper moistened with 0 15 ml of 10 N NaOH The flasks were [illegible] ubation for 10 min at 37° C. in [illegible] -C^{14} (glucose-U-C^{14})¶ was tipped [illegible] concentration of 3 5 × 10^{-3} M
The manometers were closed and oxygen consumption was measured during 2- to 4-hour incubation periods with shaking At the end of this time, 3 ml of

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TABLE 2

INHIBITION BY HYDROCORTISONE AND DESOXYCORTICOSTERONE OF ENDOGENOUS RESPIRATION, OXYGEN CONSUMPTION, AND GLUCOSE OXIDATION OF CELL SUSPENSIONS OF THYMUS AND LYMPHOSARCOMA

	Hydrocortisone*		Desoxycorticosterone*	
	Thymus	Lymphosarcoma	Thymus	Lymphosarcoma
Endogenous respiration	16(1)†	44(1)	46(1)	65(2)
Oxygen consumption‡	32(8)	55(2)	86(2)	66(2)
Glucose oxidation	31(8)	69(2)	93(2)	88(2)

Val-

Tissue from adrenalectomized animals was used in initial experiments with the belief that more uniform data might be obtained. The data are expressed as the percentage change observed in the extent of glucose oxidation and oxygen consumption under identical conditions, with and without the addition of steroid. The data indicate that the addition of either hydrocortisone or desoxycorticosterone *in vitro* inhibits significantly the oxygen consumption and glucose oxidation by mesenteric lymph nodes of adrenalectomized rats. The results of this experiment suggest that desoxycorticosterone is somewhat more effective than hydrocortisone in producing this inhibition. This is supported by other data (see below).

Comparative effects of hydrocortisone and desoxycorticosterone on glucose oxidation by thymic and lymphosarcoma cell suspensions. The metabolism of the lymphosarcoma cells appeared to be more sensitive to the presence of hydrocortisone or desoxycorticosterone than did that of cells of normal lymphoid tissue (TABLE 2). With the quantity of steroid employed, namely, 180 μg, hydrocortisone produced 2 to 3 times as great an inhibition in endogenous respiration, oxygen consumption, and glucose oxidation of lymphosarcoma cells as was seen with thymic lymphocytes. The inhibitory effects of desoxycorticosterone were significantly greater than those of hydrocortisone for both the normal and the malignant lymphocytes. The data support those of TABLE 1, which suggest that the concentration of desoxycorticosterone required to cause a given degree of inhibition of glucose oxidation and oxygen consumption by normal, as well as by malignant, lymphoid tissue is lower than that for hydrocortisone. The data also suggest that the lymphosarcoma cells are generally more susceptible to the inhibitory actions of the steroids than are thymic lymphocytes.

Effects of steroid injection in vivo on glucose oxidation by lymphocytes in vitro. The inhibitory effects of hydrocortisone and desoxycorticosterone on the oxidation of glucose by thymic and mesenteric lymphocyte cell suspensions were also seen in experiments in which the steroid was injected 4 hours prior to removal of the lymphoid tissue for study *in vitro*. These data are shown in TABLE 3. The uniformity of the data obtained for glucose oxidation by thy-

determination of radioactivity Nucleic acid extracts were shaken repeatedly with ether to remove trichloroacetic acid, then directly plated on stainless steel planchets for counting

Experiments were conducted to obtain evidence that the radioactivity incorporated into the protein and nucleic acid fractions represented actual synthesis A possible "exchange" phenomenon was ruled out by experiments in which cells were labeled by incubation with glycine-2-C^{14}, washed free of radioactive glycine, then reincubated in the presence of a large excess of nonradioactive glycine, the specific activities of cellular proteins and nucleic acids were not decreased by this procedure In addition, specific activites of isolated cellular proteins were unchanged by treatment with mercaptoethanol, performic acid, or alkali, this minimized the possibility that glycine could have been incorporated into small molecules, that is, into glutathione, which can bind to protein by means of disulfide linkages

In each experiment conducted throughout all these studies aliquots of the same lymphoid tissue slices or lymphoid cell suspension were used with and without steroid and compared with one another under identical experimental conditions at the same time in order to minimize experimental variables

Results

Influence of hydrocortisone and desoxycorticosterone on glucose oxidation by mesenteric lymph nodes The data obtained from study of the effect of hydrocortisone and desoxycorticosterone on the aerobic oxidation of glucose-U-C^{14} by mesenteric lymph nodes of adrenalectomized rats are shown in TABLE 1.

TABLE 1
INHIBITION BY HYDROCORTISONE AND DESOXYCORTICOSTERONE OF GLUCOSE-U-C^{14} OXIDATION AND OXYGEN CONSUMPTION OF MESENTERIC LYMPH NODES OF ADRENALECTOMIZED RATS

Incubation time, hours	Steroid	Amount, μg	Mean per cent inhibition of glucose oxidation	Mean per cent inhibition of oxygen consumption
2	H*	20(1)†	17	23
	H	40(6)	20 (11–33)‡	20 (9–40)
	H	80(1)	27	27
3	H	20(1)	2	0
	H	40(6)	31 (13–43)	26 (13–37)
	H	60(4)	36 (33–40)	25 (12–43)
	H	80(5)	34 (25–41)	24 (13–38)
2	D	40(4)	33 (25–39)	36 (16–53)
3	D	20(1)	46	22
	D	30(3)	18 (7–30)	8 (0–24)
	D	40(1)	80	70

TABLE 4
EFFECTS OF CERTAIN POTENT GLYCOGENIC STEROIDS ADDED *IN VITRO* ON ENDOGENOUS RESPIRATION, OXYGEN CONSUMPTION, AND GLUCOSE OXIDATION BY THYMIC LYMPHOCYTE SUSPENSIONS*

Steroid	Endogenous respiration	Oxygen consumption†	Glucose oxidation
Cortisone	26	15	20
Hydrocortisone	16	32	30
Δ^1-Hydrocortisone	12	32	41
9α Fluorohydrocortisone	20	16	30
6-Methyl Δ^1-hydrocortisone	10	17	27

* Values given are per cent inhibition produced by 180 μg of steroid as compared to controls without steroid
† With added glucose

TABLE 5
INFLUENCE OF C-21 HYDROXYL GROUP OF VARIOUS STEROIDS ADDED *IN VITRO* ON INHIBITION OF ENDOGENOUS RESPIRATION, OXYGEN CONSUMPTION, AND GLUCOSE OXIDATION BY THYMIC LYMPHOCYTES*

Steroid	Endogenous respiration	Oxygen consumption†	Glucose oxidation
Hydrocortisone	13	32	30
Hydrocortisone-21 acetate	8	13	30
Desoxycorticosterone	46	86	93
Desoxycorticosterone acetate	21	40	39
11-Desoxycortisone	23	17	12
11-Desoxycortisone acetate	3	2	2

* Values given are per cent inhibition produced by 180 μg of steroid as compared to controls without steroid
† With added glucose

nificantly enhance biological effectiveness (for example, introduction of a double bond between C-1 and C-2, or of a fluorine atom at C-9, or substitution of a methyl group at C-6) do not produce comparable enhancement of activity or even significant changes in the ability of a steroid to inhibit the endogenous respiration, oxygen consumption, or glucose oxidation of thymic lymphocyte suspensions

The data in TABLE 5 suggest that a free hydroxyl group in position 21 of the steroid nucleus may contribute to the inhibitory potency of a steroid under the experimental conditions employed On the other hand, the high activity of progesterone (TABLE 6) indicates that a methyl group as C-21 does not significantly alter the inhibitory potency of a steroid The data in TABLE 6 also reveal that a strikingly uniform diminished inhibitory activity of C-21 steroids is observed when an α-hydroxyl group at C-17 is introduced into the molecule This is seen in comparisons of effectiveness of corticosterone versus hydrocortisone, desoxycorticosterone versus 11-desoxycortisone, and progesterone versus 17-α-hydroxyprogesterone

The data in TABLE 7 reveal that not only C-21 steroids but C-18 and C-19 compounds as well exert an inhibitory influence on lymphocyte metabolism

TABLE 3

EFFECT OF *IN VIVO* INJECTION OF STEROIDS ON GLUCOSE-U-C[14] OXIDATION BY THYMIC AND MESENTERIC LYMPH NODE CELL SUSPENSIONS *IN VITRO*

Type of animal	Route of injection	Type of cells studied	Steroid injected	μ Atoms of glucose carbon oxidized/5 × 10⁸ cells	Inhibition per cent
Normal	Intraperitoneal	Thymus	—	3.54	—
			H*	2.30	35
			D	2.64	25
Adrenalectomized	Subcutaneous	Thymus	—	3.54	—
			H	2.76	22
Normal	Intraperitoneal	Mesenteric lymph nodes	—	3 33	—
			H	1 34	60
			D	2 44	27
Adrenalectomized	Subcutaneous	Mesenteric lymph nodes	—	3.54	—
			H	1 53	57

* H = hydrocortisone, D = desoxycorticosterone

mic or mesenteric lymphocyte suspensions from normal or adrenalectomized rats may be noted, it indicates the reproducibility of the experiments. Hydrocortisone injection into normal or adrenalectomized rats produced an average inhibition of approximately 28 per cent in glucose oxidation *in vitro* by thymic cells and, when mesenteric lymphoid cells were studied, an inhibition of approximately 60 per cent. Desoxycorticosterone injection produced an average inhibition of glucose oxidation of approximately 26 per cent for both thymic and mesenteric cells.

The data indicate that whatever may be the mechanism of the in vitro inhibitory effects of hydrocortisone and desoxycorticosterone on lymphocyte metabolism, similar metabolic influences are exerted by these steroids on the lymphoid tissue of the intact animal This is noteworthy particularly for desoxycorticosterone which, in the dose used, has been reported[2, 3] to be without influence on lymphoid tissue size or structure

Influence of steroid structure on respiration and oxidation of glucose by thymic cell suspensions Cell suspensions of rat thymus glands were employed in this study because of their ease of preparation in uniform quantity Each steroid

were con-
data ob-
the con-
changes
s, a diminution in the control levels of endogenous respiration, oxygen consumption, and glucose oxidation

In TABLE 4 are assembled data for certain of the diverse steroids that have

TABLE 8
RELATIVE RATES OF INCORPORATION *IN VITRO* OF RADIOACTIVITY FROM GLYCINE-2-C^{14} INTO TOTAL PROTEIN AND NUCLEIC ACIDS OF THYMIC AND LYMPHOSARCOMA CELL SUSPENSIONS*

Cells used	Proteins	Nucleic acids
Thymus, intact rats	100	100
Thymus, adrenalectomized rats	78	80
Lymphosarcoma, intact rats	23	37
Thymus, lymphosarcoma bearing rats	77	75

* The rates for normal thymus lymphocytes are used as the reference standard and are arbitrarily given as 100 in this table

gree of steroidal structural specificity with respect to the inhibitory phenomena measured, and (3) that certain steroids which, in the quantities used, do not alter lymphoid tissue size or histology may, nevertheless, when administered *in vivo*, lead to *in vitro* demonstrable metabolic effects in lymphocytes obtained from the steroid-injected animal

Incorporation in vitro of radioactivity from glycine-2-C^{14} into proteins and nucleic acids of lymphocytes The rates of incorporation *in vitro* of radioactivity from glycine-2-C^{14} into the total proteins and total nucleic acids of lymphocyte cell suspensions have been established over 2- to 3-hour periods at 37° C, with gentle shaking in air In TABLE 8, the rates observed for thymic lymphocytes obtained from normal rats have been indicated as 100, for purposes of comparison In comparisons of data with thymic lymphocytes from normal and adrenalectomized rats, it will be seen from TABLE 8 that, for thymic lymphocytes from adrenalectomized rats, the rate of incorporation of radioactivity into proteins and into nucleic acids is approximately three-fourths that of the rate with lymphocytes from normal rats Lymphocyte suspensions prepared from the transplantable lymphosarcoma incorporated radioactivity into their proteins at a rate one quarter that seen with thymic cells, and at a rate into nucleic acids approximately one third that of thymic lymphocyctes from normal rats The data suggest that the presence of a growing lymphosarcoma in a normal rat causes a slight decrease in the rate of incorporation of radioactivity from glycine-2-C^{14} into the proteins and nucleic acids of the thymic cells obtained from a tumor-bearing host

Influence of various steroids on incorporation in vitro of radioactivity from glycine-2-C^{14} into proteins and nucleic acids of lymphocytes A variety of steroids have been studied in varying concentrations in an effort to assess their influence on the incorporation phenomena that have been described [illegible]

[illegible]

without steroid addition

It may be noted again that the inhibitory effects exhibited by some steroids, for example, 17-ethyl-19-nortestosterone and 17-ethyltestosterone, are seen with exceedingly low concentrations of steroid, that is, 10^{-4} to 10^{-5} M There is an evident correlation, in most instances, of the effectiveness of a steroid in

Table 6

Effects of an α-17-Hydroxyl Group of Various Steroids Added *in Vitro* on Endogenous Respiration, Oxygen Consumption, and Glucose Oxidation by Thymic Lymphocyte Suspensions*

Steroid	Endogenous respiration	Oxygen consumption†	Glucose oxidation
Corticosterone	31	63	68
Hydrocortisone	16	32	30
Desoxycorticosterone	46	83	93
11-Desoxycortisone	*23*	*17*	*12*
Progesterone	42	65	74
17α-Hydroxyprogesterone	8	11	9

* Values given are per cent inhibition produced by 180 μg of steroid as compared to controls without steroid

† With added glucose

Table 7

Effects of Various Steroids Added in Vitro on Endogenous Respiration, Oxygen Consumption, and Glucose Oxidation by Thymic Lymphocyte Suspensions*

Steroid	Endogenous respiration	Oxygen consumption†	Glucose oxidation
Testosterone	31	29	30
17-Ethyltestosterone	87	88	97
19 Nortestosterone	65	77	93
17-Ethyl-19-nortestosterone	—‡	94	99
Estradiol	15	21	33
Cholesterol	2	2	5

[illegible] 180 μg of steroid as compared to con-

when added *in vitro* Particularly striking is the potency that appears as a result of the introduction of an ethyl group in position 17 The two compounds, 17-ethyltestosterone and 17-ethyl-19-nortestosterone, exhibited a greater degree of inhibitory activity than any other of the various compounds studied It may be noted that 17-ethyl-19-nortestosterone, like hydrocortisone and desoxycorticosterone (see above) will produce, when injected *in vivo*, evidence of the inhibitory phenomena in lymphoid tissue taken from injected animals and studied subsequently *in vitro* [20, 21] The negative findings with cholesterol (Table 7) may also be mentioned, as well as similar negative or inactive manifestations in other studies[20] with dihydrocortisone and tetrahydrocortisone acetates It will be seen that the C-18 compound may be a "nor" steroid, that is, 19 nortestosterone and 17-ethyl-19-nortestosterone, or may have an aromatic ring A, that is, estradiol

From the data presented in this section, it would appear (1) that exceedingly low concentrations, that is, 10^{-4} M, of many steroids added to lymphocyte suspensions *in vitro* will suppress metabolic activity, (2) that there is some de-

Discussion

General aspects of steroid action on lymphocyte metabolism in vitro The experimental data that have been presented indicate that a wide variety of steroids can exert a profound metabolic influence on lymphocytes *in vitro* From the 2 types of studies of lymphocyte metabolism that have been examined, that is, glucose oxidation and protein and nucleic acid synthesis, it is evident that (1) certain steroids may have a direct action *in vitro* on lymphocyte metabolism, (2) an active steroid may be effective at a very low concentration, namely, 10^{-8} M, a concentration that is less than that needed to demonstrate inhibition of respiration by certain respiratory poisons, for example, cyanide, and (3) the steroids studied exhibit a degree of specificity with respect to the process that they may influence[23] The relationships between steroid structure and activities measured have been considered in the earlier presentation of the results obtained

On the basis of available evidence it is not possible to present a mechanism for the steroidal influences that have been described At least a portion of the effects can be attributed to an action of the steroid upon processes within the lymphocyte rather than solely to action of the steroid upon the lymphocyte membrane This conclusion is derived from several aspects of the data Perhaps of greatest significance is the fact that, in specific instances, the *in vivo* administration of a steroid resulted subsequently in metabolic alterations measured *in vitro* in the lymphoid tissue of the injected animal, which were similar to those seen when the steroid was incubated directly with the lymphoid tissue *in vitro* In this connection it was of considerable interest that steroids which, in the dose used, have been described previously as having no discernible effects on lymphoid tissue size or morphology when administered to experimental animals, nevertheless, on injection, produce biochemically measurable metabolic changes in lymphoid tissue This is seen, for example, in the studies of the influence of desoxycorticosterone injection on the *in vitro* oxidation of glucose by lymphoid tissue taken 4 hours later from the injected animal

The fact that diverse steroids affect different metabolic phenomena in lymphocytes to varying degrees would also appear to argue against a membrane phenomenon (for example, permeability alterations) as the sole basis for explanation of the changes observed Thus, desoxycorticosterone added to lymphocyte suspensions *in vitro* has no effect on transaminase activity of these cells[22] The inhibition of the endogenous respiration of lymphocytes by certain steroids, in the absence of added substrate, also speaks for a steroidal influence exerted within the lymphocyte Also, the alterations produced in the transaminase activity of high-speed supernatants obtained from broken-cell preparations of lymphocytes as a result of prior incubation of the cells with hydrocortisone[22] suggest that cell membrane, substrate, and steroid need not be present simultaneously for manifestation of certain of the metabolic effects of steroids on lymphocytes Finally, experiments in progress have demonstrated similar steroidal effects *in vitro* on cell-free preparations obtained from lymphoid tissue[23a]

TABLE 9

COMPARATIVE EFFECTIVENESS OF VARIOUS STEROIDS *IN VITRO* IN INHIBITING THE RESPIRATION AND THE INCORPORATION OF RADIOACTIVITY FROM GLYCINE-2-C^{14} INTO TOTAL PROTEIN AND NUCLEIC ACIDS OF THYMIC CELL SUSPENSIONS

Steroid	Concentration, $M \times 10^{-5}$, required to produce a 50 per cent inhibition in		
	Respiration	Protien synthesis	Nucleic acid synthesis
17-Ethyl-19 nortestosterone	6 2	3 2	5 2
17-Ethyltestosterone	9 0	5 3	10 0
Desoxycorticosterone	17 0	5 0	8 5
19-Nortestosterone	42 0	15 0	27 0
Corticosterone	36 0	17.0	25 0
Testosterone	70 0	36 0	31 0
Hydrocortisone	143 0	57 0	70 0
Cortisone	>190 0	>120 0	>96.0
11-Desoxycortisone	>136 0	>160 0	>84 0

diminishing respiration and its inhibition of protein and nucleic acid synthesis This is in accord with observations[21 25 29] that metabolic inhibitors that block oxygen uptake or uncouple oxidative phosphorylation, for example, dicumarol, azide, and 2,4-dinitrophenol, also inhibit these synthetic processes. There is also some correlation of the data in TABLE 9 with the results presented above on the inhibitory effects of steroids on glucose oxidation by thymic lymphocyte suspensions It will be recalled that 17-ethyltestosterone and 17-ethyl-19-nortestosterone were the 2 most active steroids in those studies also.

It may also be mentioned that *in vivo* administration to rats of desoxycorticosterone, hydrocortisone, or 17-ethyl-19-nortestosterone at 24 hours, and again at 4 hours prior to sacrifice of the animals, with subsequent *in vitro* study of [illegible]

Other investigators have reported that the incorporation of amino acids into the proteins of thymic cell suspensions[26 27] and into the proteins and nucleic acids of isolated lymphocyte nuclei[28 29] is inhibited by cortisone or hydrocortisone Koritz and Dorfman[30 31] demonstrated that the addition of desoxycorticosterone *in vitro* inhibited the incorporation of glycine-2-C^{14} into the proteins of reticulocytes These investigators used somewhat higher concentrations of steroid than those employed in the present study It is interesting to note that, as in the case of certain of the experiments reported here, Koritz and Dorfman[30] observed an almost complete loss of inhibitory effect resulting from the introduction of a 17α-hydroxyl group Also, all steroids that were effective had an inhibitory action, in no case was there stimulation of incorporation of glycine-2-C^{14} into reticulocyte proteins Hayano, Dorfman, and Yamada[32] and McNaught *et al*[33] found that desoxycorticosterone and other steroids added *in vitro* influenced the activity of a variety of enzyme systems

diseases, infectious diseases, immunology, and special areas of metabolism. Certain of the roles of the lymphocyte in hematology, malignant diseases, and in infectious diseases are touched upon in this monograph. The remainder of the present discussion will be limited to a consideration of the role of the lymphocyte in protein and nucleic metabolism as well as in antibody production, which may be viewed as a specialized type of protein and nucleic acid metabolism.

The lymphocyte and protein and nucleic acid metabolism. The rapid involution of lymphoid tissues seen in fasting animals and its dependency upon adrenal cortical steroid secretion was studied in detail by White and Dougherty.[12,13] In mice that fasted for a forty-eight-hour period, the loss in lymphoid tissue protein was greater than that seen in total proteins of liver, commonly thought to be the chief source of readily mobilizable protein. These observations and the demonstration[1,15] of the contributions of lymphoid tissue to the blood proteins led to a suggested specialized role of lymphoid tissue in protein metabolism. It was postulated[1,12,13] that lymphoid tissue is a source of readily available or mobilizable protein, the rate of release of which from lymphoid tissue is regulated, in most instances, by the level of secretory activity of the hypophyseal adrenocorticotropic hormone. Circumstances that augment secretion of this hormone, that is, exposure to any one of a wide variety of noxious stimuli, lead to making available lymphoid tissue protein.

It was further suggested[1,12,13] that lymphoid tissue protein, either released from within lymphoid structures or transported via circulating lymphocytes, could be made available to other organs and tissues of the body for metabolic purposes. This was described as a *translocation of protein*. The propensity of lymphocytes to infiltrate into all of the body structures may add significance to the role of the lymphocyte in the translocation of protein. Andreasen[16] has suggested that the lymphocyte could have a function in protein metabolism analogous to that of the fat cell in lipid metabolism.

Whether the lymphocyte may function in a similar manner in nucleic acid metabolism remains to be established. Lymphocytes are noteworthy for their high concentration of nucleic acids. The possible role of these nucleic acids in reutilization phenomena in general and in antibody synthesis in particular will be considered below. In this sense there could occur a *translocation of nucleic acids* similar to the translocation of protein that has just been discussed. It may be noted that adrenal cortical steroids affect nucleic acid metabolism in lymphocytes. Inhibition of nucleic acid synthesis *in vitro* by the addition of hydrocortisone has been described in this presentation. Mirsky and his associates[21,22] demonstrated inhibition of the *in vitro* incorporation of labeled precursors into the nucleic acids of lymphocyte nuclei by the addition of cortisone *in vitro*. Shortly following injection of an 11-oxy adrenal cortical steroid (for example, cortisone) there is an inhibition of mitosis in the lymphocytes of lymphoid structures.[2,3] This is reflected in a diminished rate of incorporation of radioactive phosphorus into the nucleic acids of these cells.[22,23]

The shedding or budding of lymphocyte cytoplasm is known to occur normally in the life of the lymphocyte and is seen to a greatly enhanced degree

The possibility may also be considered that the described inhibitions by steroids *in vitro* of the several metabolic processes studied in lymphocytes are manifestations of a "toxic" action of these steroids on the cells Trowell[34] has described the exquisite sensitivity of lymphocytes to the presence of exceedingly low concentrations of cortisone. When added to lymphocytes in tissue culture, this steroid in a concentration of 3×10^{-7} M caused death of some cells at the end of a 5-hour incubation period with steroid, as evidenced by the pyknotic appearance of the cells.

In the present studies, incubation periods were for a maximum of 3 hours Cell counts were conducted at the beginning and at the end of the incubation periods, with careful examination of cell morphology and staining propensity with Saffranin O in Tyrodes' solution[35] Viewed under the microscope, with or without dye, cell numbers and appearance gave some assurance that the added steroids were not causing cell destruction Moreover, more positive evidence for viability of these lymphocytes was afforded by the following observations.[21]

A lymphocyte suspension was incubated for 2 hours with desoxycorticosterone and the inhibitions of respiration and C^{14}-incorporation into proteins and nucleic acids were obtained as described previously. The incubation mixture was centrifuged and the lymphocytes washed twice with physiological saline to free them of incubation medium On resuspension in fresh buffer medium with glycine-2-C^{14}, these cells showed rates of respiration and of incorporation of C^{14} into lymphocyte proteins and nucleic acids that were characteristic of lymphocyte suspensions without added steroid The findings argue strongly against a "toxic" effect of the steroid on lymphocytes under the experimental conditions employed

Comparison of the effects of steroids on normal and malignant lymphocytes in vitro Several interesting differences are apparent on examination of the data obtained with lymphocytes from thymic tissue, as compared with those obtained from cells of the transplantable lymphosarcoma In the studies of glucose oxidation to carbon dioxide, the metabolism of the lymphosarcoma cells appeared to be more sensitive to the presence of hydrocortisone than did normal lymphocytes That is, a given amount of the steroid produced a significantly greater inhibition of glucose oxidation by lymphosarcoma cell suspensions than was the case for suspensions of thymic cells Similar data were obtained when desoxycorticosterone was the steroid used

The rate of *in vitro* incorporation of radioactivity from glycine-2-C^{14} into the total proteins and nucleic acids of lymphosarcoma cell suspensions is less than that of normal thymic lymphocytes

Cell suspensions of the transplantable lymphosarcoma exhibit a significantly higher basal level of transaminase activity than do normal lymphocytes,[23] and this enzymic activity is unaffected by *in vitro* addition of hydrocortisone This is in marked contrast to the responsiveness of the transaminase activity of normal thymic lymphocytes to the presence of hydrocortisone[23]

Aspects of Lymphocyte Function

The problem of the functions of the lymphocyte has been one that has stimulated a variety of investigations in diverse areas hematology, malignant

The lymphocytosis observed by Roberts and White[42] with thymic tissue protein fractions could be related to the lymphocyte-stimulating effects reported by others. Parsons, Gulland, and Barker[44] observed a lymphocytosis in mice receiving injections of pentose nucleotides. The lymphocytosis activity seen by Roberts and White was concentrated in thymus fractions rich in nucleic acid or nucleoprotein. In important studies, Metcalf has provided evidence, in this monograph and elsewhere,[45, 46] that the thymus secretes a factor that, when injected intracerebrally into immature mice of certain inbred strains, produces a lymphocytosis. The active material is reported to be nondialyzable and heat labile and is termed lymphocytosis-stimulating factor by Metcalf.

Role of Lymphoid Tissue in Antibody Production

A number of excellent reviews of the proposed sites and mechanisms of antibody formation is available.[47-60] This discussion will therefore refer to only a small portion of the pertinent literature, and will devote more attention to specific points.

A role for cells of the reticuloendothelial system in antibody formation has been accepted for more than fifty years, based upon initial studies of many investigators, including Metchnikoff,[51] Pfeiffer and Marx,[52, 53] and the Wassermanns,[54, 55] and extended to a significant degree later by Sabin.[56] For almost an equal number of years it has been known that lymph nodes play a role in infectious processes (compare McMaster[49]). Subsequently, numerous investigators, notably Murphy and his colleagues,[57, 58] established the importance of lymphoid tissue in antibody formation by demonstrating that reduction of this tissue in animals by X rays depressed antibody production. In contrast, experimental stimulation of lymphoid tissue proliferation caused an increase in antibody production.[59] These correlations between lymphoid cell numbers and the capacity to form antibody, as well as the demonstration by Murphy and his collaborators[57] of the role of the lymphocyte in the immunity of animals to transplanted tumors, have formed the basis for subsequent investigations in the exceedingly important field of heterologous tissue transplants. Treatment of the host by an experimental procedure that either diminishes the quantity of functioning lymphoid tissue, for example, adrenal cortical steroid administration[60] or X radiation,[60, 62] provides an animal that is significantly more receptive to heterologous tissue transplants than is the case with untreated animals. This has proved of great importance in studies of experimental tumor transplantation,[63] and it is assuming suggestive significance in connection with possible therapeutic use of heterologous transplantation in the human. The efficiency of heterologous transplantation in immature animals with inadequately developed lymphoid structures may also be noted in relation to the role of lymphoid tissue in immune phenomena.[64-66]

The role of regional lymph nodes in localized antibody production was clearly established by the precise experiments of McMaster and Hudack.[49, 67-70] By administering two different antigens (one into each ear) to mice, it was possible to demonstrate that the antibody to the antigen injected in one ear appeared first only in the lymph node draining that ear, while antibodies were

in lymphocytes of lymphoid tissues and of blood shortly following administration of adrenal cortical steroid[2,3] This phenomenon was postulated by White and Dougherty[1,15] to be a mechanism for release of cytoplasmic constituents. This loss of cytoplasmic material was seen often to lead to the *presence of cytoplasmic-free lymphocyte nuclei in lymph node imprints of* hormone-treated animals[2,3] It was suggested that the shed cytoplasmic substance might be reutilized by other cells, including reticulum cells, macrophages, and lymphocytes In the case of cytoplasm derived from lymphocytes of immunized animals, reutilization of cytoplasmic components by primitive cells could be a mode of "sensitizing" these cells Thus subsequent development of lymphoid cells from these cells by the mechanism described below might result in lymphocytes that contain antibody. It was also pointed out[1,3] that the apparent morphologic integrity of a number of the lymphocyte nuclei remaining following shedding of cytoplasm created the opportunity for reutilization of such nuclei for formation of new lymphoid cells The hypothesis for a reutilization of lymphocyte constituents has been extended by the investigations of Hamilton[39,41] and of Trowell,[42] and further evidence for the reutilization phenomenon among lymphocytes has been provided by both of these investigators in this monograph The role of the lymphocyte in antibody production (see below) also could include a reutilization mechanism Under appropriate conditions, "information-carrying" portions of antibody-containing lymphoid cells (for example, a desoxyribonucleic acid component) acting as a donor, may incite antibody formation or lead to other expressions of immune phenomena by being transferred to appropriate recipient lymphoid cells The susceptibility of this concept and other aspects of the postulated mechanism of antibody formation to experimental study is being examined

The release of lymphocyte proteins from lymphoid cells as a consequence of augmented blood levels of certain adrenal cortical steroids suggested a study of the nature of the proteins of the lymphocyte Moreover, an additional working hypothesis for the examination of thymus proteins was based on the well-established interactions between endocrine glands and the activity of their target organs Demonstration that the lymphocyte is an end cell of adrenal cortical steroid action raised the possibility that lymphocyte constituents, in turn, might influence the secretory activity of the adrenal cortex, that is, they might suppress that activity Roberts and White[43] fractionated an alkaline (*p*H 7 6) aqueous extract of calf thymus with cold ethanol The initial extract contained at least 7 components, as revealed by electrophoretic analysis, proteins possessing the mobility of serum beta- and gamma-globulins were present Ethanol-insoluble fractions were obtained at 10, 20, 30, and 40 per cent alcohol concentrations The fraction insoluble at 40 per cent [illegible] nucleic acids and, when injected [illegible] an elevation in the blood lym- [illegible] lt rats in an amount of 10 mg per day for 13 days, the 40 per cent ethanol insoluble fraction produced a significant increase in weight of the thymus, with a depression in adrenal weight

both the plasma cell and the lymphocyte function in antibody synthesis. The influence of adrenal cortical steroids on these processes will be indicated.

The plasma cell and antibody production A review of the role of the plasma cell in the production of hyperglobulinemia was presented by Fagraeus[72] in 1948 Since that time a number of investigators have believed that they have localized antibody production in plasma cells However, as indicated previously, the possible relationship of true plasma cells to antibody formation is somewhat obscured by the common practice of designating any cell of the lymphoid series that has a basophilic cytoplasm as a "plasma cell" Despite this, evidence has accumulated that strongly implicates plasma cells as intimately concerned with antibody synthesis Ehrich[73, 74] and his associates and Dixon and his colleagues[47] have assembled a large quantity of significant data supporting a role for the plasma cell in the elaboration of antibody The development of antibody titer under a variety of experimental conditions could be correlated with the number of plasma cells found at sites of antibody synthesis, whether these were in lymphoid structures or in subcutaneous depots into which antibody-containing lymphocytes were transplanted

Coons and his associates[75, 76] have been particularly active in more recent efforts to define the precise cell types concerned with antibody production Using the fluorescent antibody technique elaborated in their laboratory, these investigators have provided evidence that, in the hyperimmune rabbit, antibody is present in groups of plasma cells in the red pulp of the spleen and in the medullary areas of lymph nodes In studies of tissues taken from animals shortly after the injection of a second antigenic stimulus, it was concluded[77] that the first cells to contain antibody "are large cells with a thin rim of basophilic cytoplasm During the 2 or 3 days after their first appearance they multiply, synthesize antibody specific for the antigen which stimulated their development, and differentiate through immature to mature plasma cells"

In a recent study of the cellular localization of normal gamma-globulin, Ortega and Mellors[80] have extended the observations of White[51] which demonstrated the presence of antibody in plasma cells containing Russell's bodies obtained from immunized mice and rabbits Ortega and Mellors,[80] utilizing the fluorescent-labeled protein technique, believe they have obtained evidence for three catagories of cells that contain normal gamma-globulin Two of these catagories were described as subdivisions of plasma cells, one contained conspicuous Russell's bodies and the other group was composed of mature and immature plasma cells without Russell's bodies The third category of gamma-globulin-containing cells was found in germinal centers of lymphatic nodules and was composed of what were described as large and medium lymphocytes and primitive reticular cells No gamma-globulin was observed in mature lymphocytes, mast cells, neutrophilic and eosinophilic granulocytes, reticulum cells, free histiocytes, or erythrocytes The authors also postulated that the Marschalko-type plasma cells are derived from a primitive form resembling a large lymphocyte

The lymphocyte and antibody formation The presence of antibody in lym-

not detectable in the ear tissue. Similar data were obtained for the lymph node and ear tissue of the other ear, relative to the antigen injected into that ear. This study thus ruled out the possibility that demonstration of antibody in a regional lymph node might be due to selective concentration of antibody formed elsewhere in the organism.

The lymphocyte versus the plasma cell in antibody formation Demonstration of antibody formation in lymph nodes has led to the question of the precise cell type concerned with production of the immune globulins The lymphocyte and the plasma cell have been the most frequently implicated morphologic units, the favor of experimental opinion falling alternatively on one or the other of these cells, and the same investigators exhibiting some degree of shifting of their favoritism from the lymphocyte to the plasma cell, then back again to the lymphocyte The question of which cell type is primarily concerned with antibody formation may be resolved with the broader point of view that both the lymphocyte and the plasma cell are capable of immune globulin synthesis It may be pointed out that certain of the evidence regarding both the site and mechanism of antibody formation has been obscured by controversial interpretations due to failure of complete agreement by all investigators on two fundamental questions These are (1) Is the term plasma cell too loosely used in its application to all lymphocytes with basophilic staining cytoplasm, or should this designation be reserved for the typical plasma cell of Marschalko with the characteristic perinuclear hof, or is the plasma cell a special form of the lymphocyte? and (2) What are the genealogical relationships among the cells characterized as reticuloendothelial cells?

The first question is of significance in relation to the cell type concerned with antibody production, since the circumstances associated with augmented production of immune globulins are characterized frequently by increased numbers of basophilic staining lymphocytes in lymphoid structures These have been described as "plasma cells" by some investigators and as basophilic-staining lymphocytes by others

The second question is of fundamental importance in relation to the mechanism of antibody formation The phagocytic role of reticuloendothelial cells *as a first requisite for initiation of primary antibody production is universally* acknowledged Visualization of the mechanism by which the engulfment and alteration of antigen lead to antibody appearance in lymphocytes (see below) or plasma cells is seriously influenced by the point of view held with respect to the cellular origin of lymphocytes and whether lymphocytes may become phagocytic The multipotentiality of the lymphocyte has been established [71] These problems have also been considered in this monograph by Trowell and by Rebuck, the latter author has confirmed the multipotentiality of the lymphocyte in the human subject

It is not proposed to attempt to present a complete discussion of the data pertinent to the above two questions Rather, the remainder of this discussion will review briefly the role of the plasma cell in antibody production and will present a point of view regarding (1) the participation of the lymphocyte in antibody formation and release, and (2) a postulated mechanism by which

studies of transfer of cutaneous hypersensitivity. These investigations also afforded further substantiation of the presence of specific antibody in lymphocytes obtained from animals injected with an antigen

The demonstration of specific antibody in the lymphocytes of an immunized animal poses the question of whether the lymphocyte is capable of synthesizing antibody. The presence of antibody in lymphocytes could find explanation in a mechanism of antibody formation that envisages the lymphocyte as a cell type deriving from a precursor antibody-containing cell This is considered in the following section

Postulated Cellular Mechanisms for Antibody Formation

Heteropoietic mechanism A mechanism of antibody formation that may be termed an *heteropoietic or heteroplastic* mechanism can be postulated, based upon the above discussion and on the extensive studies of Dougherty and White on the hypophyseal-adrenal cortical influence on lymphoid tissue structure and function.[7,8] A single injection of cortisone produced within a few hours a dissolution of lymphocytes within lymphoid structures *At this time, there was also evident an increase in immature plasma cells* Small lymphocytes were evident, with an increased degree of basophilia, suggesting that hormone injection had led to the development of greater numbers of small lymphocytes containing an augmented concentration of cytoplasmic nucleoprotein The histological picture in the lymphoid structures shortly following a single injection of an adrenal cortical steroid resembles strikingly that seen in these tissues following administration of a bacterial antigen [105]

The appearance of basophilia in the cytoplasm of small lymphocytes occurs at a time when there is an elevation in the gamma-globulin fraction of the blood proteins In immunized animals, specific antibody may be demonstrated in washed lymphocytes obtained from lymphoid tissues,[82, 83, 101, 106] and antibody may be released to the blood as a consequence of lymphocyte dissolution produced by the administration of a potent adrenal cortical steroid [106] Although there is disagreement among investigators (Fischel[107]) regarding the reproducibility of the antibody release phenomenon, the anamnestic reaction, the findings have been confirmed by Hammond and Novak[108] and by Dolowitz *et al* [109] Some of the discrepancies in findings may be due to the variation in data, which may result from a single injection of cortisone to previously immunized animals, releasing preformed antibody from antibody-containing lymphocytes, as contrasted with prolonged administration of the steroid, which results in marked diminution in the numbers of antibody-producing cells Moreover, marked discrepancies may exist between antibody titers found in antibody-synthesizing tissues and the level of circulating antibody [19] The balance that obtains among the rates of antibody synthesis, antibody release from the sites of production, and antibody removal from the circulation, and the influence of the adrenal cortex on these processes has been discussed in detail [19]

The recent confirmation of earlier findings of Dougherty and White by Dolowitz *et al* [109] in man is of interest These investigators studied five cases of hypogamma-globulinemia A single intramuscular injection of Δ^1-hydro-

phocytes obtained from immunized animals has been demonstrated by a number of investigators in a variety of experimental approaches[1, 15, 82 87] These lymphocytes have been obtained from regional lymph nodes, as well as from organs containing significant numbers of lymphocytes (for example, the spleen).

The relative role of the spleen and other lymphoid organs in antibody production is determined to a significant degree by the route of antigen administration The importance of the spleen *in vivo* in antibody production following intravenous administration of antigen has been known since 1898[52-54] Antibody production by isolated portions of splenic tissue was described more than 40 years ago[98] and has been amply confirmed.[18, 49, 72, 89-98] The study of Roberts, Adams, and White[18] demonstrated the relative degree of antibody production in splenic tissue as compared to lymph nodes, depending on the route of antigen administration A single intravenous injection of antigen led to peak antibody production *in vitro* by splenic tissue When the antigen was given intraperitoneally, splenic tissue exhibited no antibody production when studied *in vitro*, whereas surviving lymphoid tissue showed a high, selective antibody production *in vitro* after intraperitoneal administration of antigen

The experiments of McMaster and Hudack[49 67-70] establishing antibody production in regional lymph nodes have been referred to above Rich, Lewis, and Wintrobe[99] had also directed attention to a possible role of lymphoid cells in immunity The careful studies of Ehrich and his colleagues[83, 100-103] initiated an era of study of the relationship of the lymphocyte to antibody production In extensive studies, thes [illegible]
foot pads of rabbits and examined [illegible]
from the draining popliteal node an [illegible]
in the popliteal node during active antibody production It was concluded that lymphocytes from an antibody-producing node contained antibody Antibody was shown to pass from cells of the efferent lymph to the lymph itself, but not in the reverse direction In considering the relationship of phagocytosis to the presence of antibody in lymphocytes found in efferent lymph draining a popliteal node into which the antigen had been introduced, Ehrich and his associates hypothesized that phagocytosis, with digestion of antigen, prepares a solubilized form of the antigen that may then enter the lymphocyte and stimulate antibody production It may be noted that the nature of this hypothesis was influenced by the opinions of the authors regarding the independent stem-cell origin of the macrophage and the lymphocyte

At the time that these investigations were being made, Dougherty, Chase, and White[82] described the presence of antibody in lymphocytes obtained from regional lymph nodes of immunized mice No antibody was found in extracts of lymphocytes from nonimmunized mice However, proteins with electrophoretic mobilities characteristic of normal serum beta- and gamma-globulins were found in lymphoid tissue extracts of normal rabbits[15] Kass[104] also demonstrated the presence of gamma-globulin in lymphocytes obtained from man, as have Ortega and Mellors[80]

The presence of antibody in lymphocytes obtained from animals injected with antigenic materials has been utilized by Chase[84 85 86] in his important

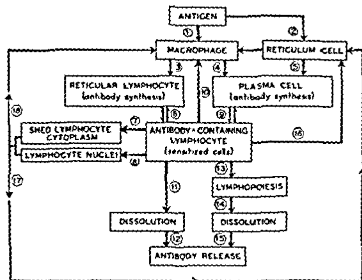


FIGURE 1.—Postulated cellular relationships in antibody formation. Heteropoietic mechanism, steps 1 through 10 and 16 through 18. Homopoietic mechanism, step 13. Steps 16 through 18 represent reutilization pathways for continuing antibody production in the absence of antigen. Acute administration or release of adrenal cortical steroids augments the rate of steps 1 through 12 and 16 through 18, and retards step 13. Continuing dissolution or involution of lymphoid structures produced by a variety of agents (for example, prolonged administration of adrenal cortical steroids or X rays) will *depress* antibody production by both the heteropoietic and homopoietic pathways, chiefly as a result of suppression of differentiation (steps 1 through 6 and step 9), and of proliferative phases (step 13), and by diminution of numbers of cell types concerned with antibody production.

could provide a basis for continuation of the antibody-forming process, in the presence or absence of antigen, and reflect a specialized case of the reutilization concept. This postulated cellular mechanism for antibody formation is depicted in FIGURE 1.

Elsewhere in these pages Speirs has provided comprehensive data regarding the role of the eosinophil in antigen-antibody phenomena. On the basis of his data, Speirs has presented his concept of antibody production. This envisages initial disruption or digestion of antigenic material by the polymorphonuclear leukocyte. The active antibody-provoking antigenic substance may then come in contact with the eosinophils; this results in a "sensitization" of the surface of the eosinophil so that there is imprinted thereupon the "template" or enzymic mechanism for the synthesis of antibody. Phagocytosis of these eosinophils by macrophages in body tissues or fluids will lead to the eventual *return of the antibody-forming mechanism back to the reticulum,* from which cells containing antibody may develop.

Whether the polymorphonuclear leukocyte and the eosinophil are to be added to the line of cellular events depicted in FIGURE 1, or whether this diagram provides an adequate mechanism for antibody formation remains for future experiments to establish. The hypothesis presented in the diagram

cortisone produced within 4 hours an elevation in the serum gamma-globulin fraction, with the values being raised to normal. This increased gamma-globulin level was maintained for three weeks; it then began to fall toward prehormone-treatment values Another injection of steroid again raised the gamma-globulin concentration toward normal. One patient is reported to have been treated with hormone every three weeks during an eighteen-month period During this period, the gamma-globulin concentration remained normal, and the patient was free of a previously persistent sore throat. It is suggested[109] that hypogamma-globulinemia and inadequate capacity to produce antibodies may be of two types In one, the size and activity of lymphoid tissue may be inadequate. This is the hypolymphatic type described by Good,[110] and may be produced experimentally by a variety of procedures that cause generalized lymphoid tissue involution, with attenuation of immune mechanisms This has been discussed previously in relation to heterologous tissue transplantation. In the second type of hypogamma-globulinemia, there may be present adequate lymphoid structures or a hyperplasia of the lymphoid tissue, with failure of a normal release or secretion of gamma-globulin by the lymphoid cells

A sequence of cellular events involved in antibody formation may now be postulated The excellent review of Sundberg[111] provides a basis for consideration of possible interrelationships among cell types concerned with antibody production The initial process is one of phagocytosis of antigen, soluble or particulate, by the phagocytic cells of the reticuloendothelial system, the histiocytes or macrophages, this process has been documented adequately since the time of Metchnikoff The questions of whether macrophages may synthesize antibody or whether lymphocytes, under special circumstances, may exhibit phagocytic capacity are left unanswered It is postulated that the macrophage may be transformed into plasma cells, as well as into basophilic-staining lymphocytes, the reticular lymphocytes[112] The latter cell also arises from primitive reticular cells[111, 113] and may also manifest phagocytic proclivity It is also likely that the plasma cell may derive from the reticular lymphocyte, as well as from lymphocytes Coons and his associates,[75, 76] using their fluorescent technique for localization of antigen in cells, found injected antigen in phagocytic cells such as Kupffer's cells, in the reticulum cells lining the lymphoid and splenic sinuses, and in developing lymphocytes in the germinal centers in the spleen and lymph nodes Development of plasma cells from the reticulum was noted

Synthesis of antibodies may occur in plasma cells or in reticular lymphocytes, both cell types may further develop into antibody-containing lymphocytes As discussed above in considerations of the role of the lymphocyte in protein metabolism, the cytoplasm shed by such lymphocytes could provide material for re-entry into reticulum cells Such re-entry might be achieved

ticulum cells could "sensitize" the latter, so that cells developing subsequently, for example, lymphoid cells, would contain specific antibody These events

ies, utilizing some form of tissue culture technique, have reported increases in antibody titers concomitant with the development of an increased number of cells with basophilic cytoplasm. More recent experimental demonstrations of antibody synthesis by lymphoid cells are those by Leduc, Coons, and Connolly,[77] by Steiner and Anker,[114] and by Ogata et al.[117] The last-named investigators were able to demonstrate incorporation of glycine-1-C^{14} into antibody and other cellular protein fractions, using cell suspensions or cell-free homogenates from popliteal lymph nodes of previously immunized rabbits.

It may be concluded that antibody synthesis has been demonstrated for plasma cells and for lymphocytes. Whether other cell types (for example, the macrophage) can synthesize antibody is not established. Antibody formation in lymphoid cells may arise via pathways relating to differentiation of cells from the primitive reticulum, a heteroplastic mechanism, or by proliferation of antibody-containing lymphocytes, a homoplastic mechanism. The heteroplastic process of antibody formation is that which predominates, particularly under circumstances of active immunization. This mechanism includes the concept of reutilization of antibody-containing cells or of components of these cells. The homoplastic mechanism may be of significance in circumstances of accentuated lymphoid tissue proliferation and, possibly, in phenomena of active or passive sensitization by techniques involving transfer of antibody-containing cells. A reutilization process may also be operative in these circumstances.

Summary

A description is presented of experimental studies of the effects of a variety of steroids on aspects of the metabolism of rat lymphoid tissue *in vitro*. Thymus, mesenteric lymph nodes, and a transplantable lymphosarcoma have been used as sources of lymphocyte cell suspensions. Measurements have been made of endogenous respiration, oxygen consumption in the presence of added glucose, oxidation of glucose uniformly labeled with C^{14}, and the incorporation of radioactivity from glycine-2-C^{14} into the proteins and nucleic acids of lymphocytes.

A variety of steroids has been examined with regard to the effects of their addition *in vitro* to lymphocyte suspensions on the several metabolic processes studied. Many steroids were observed to cause an inhibition of these reactions. Certain of these compounds, effective when added *in vitro* to lymphocyte suspensions, were also injected *in vivo*. Lymphoid tissue obtained several hours later from steroid-injected rats shows metabolic alterations similar to those seen when the steroid was added *in vitro*. Compounds that have been reported to have no effect on lymphoid tissue size and structure in the dosages used, for example, desoxycorticosterone, do nevertheless have demonstrable influences on lymphocyte metabolism when added *in vitro* or injected *in vivo*.

Relationships have been noted between steroid structure and steroid effectiveness in inhibiting lymphocyte metabolism. Comparisons of the effects of steroids on the metabolism of normal and malignant lymphocytes reveal suggestive differences.

The role of the lymphocyte in general protein metabolism, and in special-

suggests feasible experiments for its testing, and this represents a valid reason for any hypothesis.

The role of the adrenal cortical steroids in antibody production may be indicated briefly. A single administration of one of these lymphocytolytic compounds results in a dissolution of existing lymphocytes within lymphoid or-

[illegible]

bers of immature plasma cells and of lymphocytes that contain augmented amounts of cytoplasmic nucleoprotein.[2, 3] When hormone administration is repeated for several days or for longer periods of time, lymphocytolysis exceeds the rate of lymphopoiesis, with the result that the lymphoid structures have a greatly diminished cellularity. A similar depletion of lymphoid elements may ensue by exposure of an animal to any agent that causes lymphocytolysis by a direct action (for example, X rays[3]) or by the indirect augmentation of adenohypophyseal-adrenal cortical secretion by a variety of nonspecific stimuli such as fasting[3] Under these circumstances depletion of the cellular units that play a fundamental role in antibody production leads to a reduction in the immunological responsiveness of the host.

It may also be pointed out that a single injection of adrenal cortical steroids leads to a marked increase in the numbers of macrophages in lymphoid structures[2, 3] This degree of initial activation of the phagocytic mechanism could play a role in the immune response as well as in the enhancement of resistance to certain types of noxious stimuli

Homopoietic mechanism A second mode of antibody formation that may now be considered would also aid in explanation of the presence of antibody in lymphocytes from immunized animals and might be termed antibody formation by a *homopoietic* or *homoplastic* mechanism. This visualizes antibody increments arising in lymphoid cells as a consequence of proliferation of antibody-containing lymphocytes (FIGURE 1, step 13). An experimental description of this type of antibody formation was provided by Dougherty, White, and Chase[114] Transplantation of a lymphosarcoma composed of specific antibody-containing lymphocytes to a normal, nonimmunized host led to the development of new lymphosarcoma cells that contained significant quantities of antibody It may be noted that these tumor cells[115] resemble reticular lymphocytes and that some so-called lymphosarcoma cells can have exceedingly basophilic cytoplasm Inasmuch as the lymphoid tumor studied[115] *grows only* by proliferation of previously existing malignant lymphocytes, the data indicate that division and multiplication of antibody-containing lymphocytes resulted in the formation of additional antibody This mechanism of antibody synthesis could be a basis for the host sensitization and immunity that develop following the transfer of antibody-containing lymphocytes,[61, 62, 84, 85, 86, 87] although reutilization of lymphocyte constituents (see above) might also afford an explanation for transfer of immunity via lymphocytes

A number of investigators[49, 72, 74] have reported antibody synthesis *in vitro* by lymphoid cells obtained from immunized animals, with description generally of the cell type involved as predominantly "plasma cells" These stud-

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ized aspects of protein metabolism, for example, antibody formation, is discussed. The relative roles of the plasma cell and the lymphocyte in antibody synthesis are considered. An hypothesis of antibody formation that visualizes antibody synthesis by both plasma cells and lymphocytes and that presents cellular sequences of antibody formation is discussed.

Two pathways of antibody synthesis are proposed: (1) a major pathway operating as a result of the initiating stimulus of the antigen, termed a heteropoietic or heteroplastic mechanism and based upon a sequence of cellular differentiations initiated from the primitive reticulum, (2) a pathway of antibody synthesis dependent on proliferation of antibody-containing cells, possibly occurring independently of the presence of antigen and termed a homopoietic or homoplastic mechanism. Both mechanisms include the concept of possible reutilization of antibody-containing cells or constituents of these cells.

The influence of adrenal cortical steroids on certain of these processes is discussed.

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SOME PROPERTIES OF LYMPHOCYTES *IN VIVO* AND *IN VITRO*

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In this paper I shall describe the work on lymphocytes that my colleagues

cerned with 4 more or less unrelated problems, which will be dealt with in turn

Reutilization of Lymphocytes in Lymphopoiesis

Experiments in which the blood lymphocytes were labeled in the desoxyribonucleic acid (DNA) by administration of P^{32} or adenine-C^{14} to human subjects have shown a remarkably long persistence of labeled lymphocytes in the blood.[1-7] Thus Ottesen[1] concluded that most of the blood lymphocytes had a mean life span of 100 to 200 days. Hamilton[6,7] found that 300 days after administration of adenine-C^{14} the activity of the blood lymphocyte DNA was still more than one third of the peak value; the average life span of (leukemic) lymphocytes was calculated to be 85 days, and some apparently lived for more than one year. There is some reluctance to accept such a long life span, and Hamilton has suggested that these results could be interpreted in a different way that the actual life span of the cell is short, but that the DNA is specifically reutilized, with little or no degradation, in the formation of new lymphocytes. If the DNA were degraded to purines, of course, the label would be lost by dilution

I have some cytological evidence that such a specific reutilization of lymphocyte nuclear material may in fact occur Using rat lymph nodes cultured in a synthetic medium,[8] we regularly find that after about 4 days *in vitro* many of the ordinary reticulum cells (macrophages) are apparently differentiating into large lymphocytes This is contrary to the general belief (for which there is no real evidence) that large lymphocytes develop from so-called "primitive reticulum cells" that differ from the ordinary phagocytic reticulum cells of the reticuloendothelial (RE) system I have come to believe that there is only one sort of reticulum cell that is, or can be, both phagocytic and lymphopoietic

In these cultures, and also in normal lymph nodes *in vivo*, some small lymphocytes die, and the pyknotic dead cells are phagocytozed by the reticulum cells If the same phagocytic reticulum cell (macrophage) that ingests the dead small lymphocytes *can itself subsequently differentiate into a new generation* of large lymphocytes, this obviously provides a cytological basis for Hamilton's idea of a specific reutilization of lymphocyte DNA (see FIGURE 1)

In further support of this idea, we have often found in these cultures differentiating reticulum cells that had almost reached the stage of large lymphocytes but still contained in their cytoplasm the pyknotic remains of ingested

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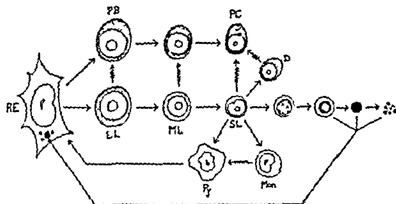


FIGURE 1. Cell interrelationships in the lymphocyte series. RE = reticuloendothelial cell, LL = large lymphocyte, ML = medium lymphocyte, SL = small lymphocyte, PB = plasmablast, PC = plasma cell, D = Downey Type I or II cell, Mon = monocyte, Py = polyblast. Stippling represents cytoplasmic RNA (basophilia). The cells are drawn to scale.

Paradoxical Resistance of Thymus Lymphocytes to High Doses of X Radiation

Lymphocytes are very readily killed by radiation, and the average radiosensitivity of any lymphocyte population can be measured accurately by counting the percentage of dead (pyknotic) lymphocytes present five hours after exposure to standard doses of radiation.[16] We have exposed rats to whole body X radiation and measured the radiosensitivity of the lymphocytes in each of the different lymphoid organs of the body.[17] We found that the different lymph nodes (lumbar, axillary, cervical, thoracic, mesenteric, ileocecal) were all equally sensitive, and that spleen was also about the same (FIGURE 2). The Peyer's patches, however, were distinctly more sensitive than any of these.

However, in the case of the thymus the results were peculiar, in that above 5000 r increasing doses gave less pyknosis. After 20,100 r there was actually less pyknosis than after 268 r. This paradoxical response of the thymus was first noted by Vogel and Ballin[15] in 1955. We have confirmed it and have also tried, without much success, to analyze it further in the following ways.

Since pyknosis is an autolytic phenomenon occurring in cells that are already dead, it seemed likely that the high doses of radiation partially inactivated the autolytic enzymes, in which case pyknosis would merely be delayed and not prevented. However, this was not the case. When the time course of pyknosis was investigated we found that after 13,400 r the percentage of pyknotic cells reached a maximum (60 per cent) at 12 hours, and that there was no further increase after 24 hours whereas, after 2010 r, all the cells were pyknotic within 11 hours. We next thought that perhaps high doses of radiation fixed some of the lymphocytes, in the histological sense, so that they could no longer autolyse. However, this was not true, either. When the thymus was removed immediately after a high dose of radiation and exposed *in vitro* to cyanide, all the lymphocytes became pyknotic and did so at the same rate as in an unirradiated control. These highly irradiated lymphocytes were also

small lymphocytes. The finding of these cells suggests to us that there is only one sort of reticulum cell that is both phagocytic and lymphopoietic

Photomicrographs of these cells have been published elsewhere[9] That is all the evidence we possess I wish to stress 2 points (1) we have shown this only *in vitro*, and (2) we cannot assess the quantitative contribution of this mechanism to total lymphopoiesis *in vivo*. We claim to have shown only that such a mechanism exists, its importance remains to be demonstrated

De Bruyn[10] and Ringertz and Adamson[11] have drawn attention to the fact that the mitotic activity in germinal centers is roughly proportional to the number of pyknotic dead lymphocytes that they contain This suggests that dead lymphocytes may be a natural stimulus to lymphopoiesis, and this would constitute a self-regulating mechanism governing the total lymphocyte population of the body.

Transformation of Small Lymphocytes to Monocytes and Macrophages

Dating from Metchnikoff (1888) there is a very impressive literature on the transformation of lymphocytes into monocytes, polyblasts, and macrophages, particularly in conditions of inflammation. In recent years Rebuck has reviewed the subject[12] and added his own very elegant experimental studies[13]

In this connection it seems worth recording that in our lymph-node cultures,[14, 15] after 4 days in our synthetic medium,[8] most of the small lympho- [illegible]

This is a regular finding Consequently, I believe that many small lymphocytes can hypertrophy in the macrophage direction, at least under certain conditions

Lymphocyte-Macrophage Cycles

At this point I would like to speculate and to introduce the concept of lymphocyte $\rightleftarrows$ macrophage cycles It seems that there may be 2 such cycles, running independently (FIGURE 1) The first of these involves the dying and ingestion of small lymphocytes by macrophages that subsequently differentiate into large lymphocytes that give rise to small lymphocytes The second cycle involves the turning of small lymphocytes into macrophages, probably via a monocyte or polyblast stage, and thence as in the first cycle

The function and significance of the lymphocyte are still entirely unknown, so I make no apology for submitting yet another hypothesis It is, at any rate, a simple one I suggest that the lymphocyte is simply a small edition of the macrophage, designed solely for circulation in the blood, and that the blood lymphocytes are simply a reserve army of potential macrophages that can be deployed rapidly in injured areas Macrophages as such do not, and indeed could not, circulate in the blood, for they would block the capillaries, especially the pulmonary ones In this light the lymphocyte may be regarded as a "pocket macrophage" designed for free passage through the capillary strains

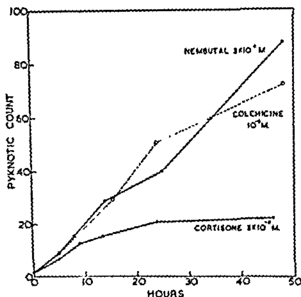
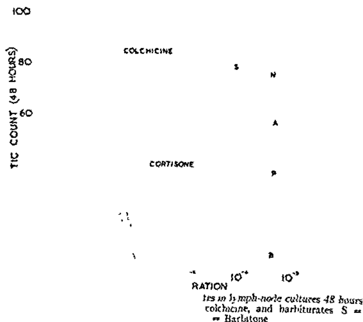


FIGURE 3 Percentage of dead (pyknotic) lymphocytes in lymph node cultures at time intervals after addition of cortisone, colchicine, or Nembutal



tes in lymph-node cultures 48 hours colchicine, and barbiturates S = = Barbitone

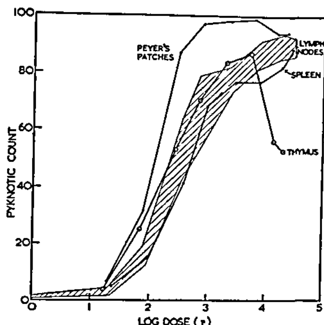


FIGURE 2 Percentage of dead (pyknotic) lymphocytes in lymphoid organs five hours after whole-body exposure to various doses of X radiation[17] Reproduced with the permission of *Radiation Research*

found to be entirely soluble in distilled water. Consequently, we think that a high dose of radiation neither fixes the cells nor inactivates the autolytic enzymes, and we have not yet found any explanation for the phenomenon

Whatever the ultimate explanation may prove to be, these experiments seem to show that "thymocytes" are not, after all, the same as small lymphocytes

Cytocidal Action of Cortisone, Colchicine, and Barbiturates on Lymphocytes in Vitro

Cortisone In 1953 I described the action of cortisone on the lymphocytes of lymph nodes cultured *in vitro*[19] Lymph nodes were cultured for 2 days, then cortisone was added to the medium, and cultures were sacrificed for study at intervals up to 48 hours It was found that cortisone killed many of the lymphocytes, and the percentages of dead (pyknotic) cells were counted The action of cortisone *in vitro* was slow, apparently much slower than *in vivo* Thus, although a few lymphocytes were killed in 5 hours, the maximum number of dead cells was found after 24 hours (FIGURE 3) By contrast, after [illegible]

[illegible]

found roughly comparable figures[20] and also showed that nitrogen mustard was highly toxic, though slightly less so than cortisone[21]

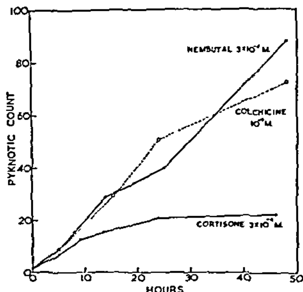


FIGURE 3 Percentage of dead (pyknotic) lymphocytes in lymph-node cultures at time intervals after addition of cortisone, colchicine, or Nembutal.

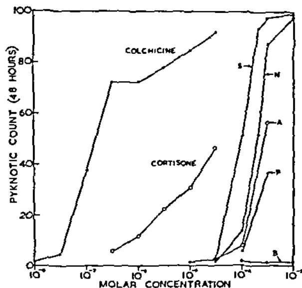


FIGURE 4 *Percentage of dead (pyknotic) lymphocytes in lymph node cultures 48 hours after addition of various concentrations of cortisone, colchicine, and barbiturates* S = Seconal, N = Nembutal, A = Amytal, P = Pentothal, B = Barbitone

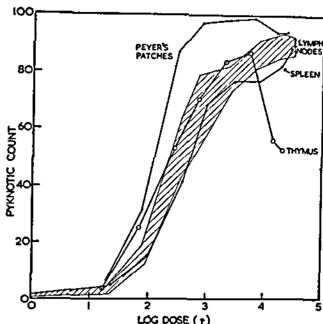


FIGURE 2 Percentage of dead (pyknotic) lymphocytes in lymphoid organs five hours after whole-body exposure to various doses of X radiation[17] Reproduced with the permission of *Radiation Research*

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at and above which Seconal and Nembutal kill lymphocytes *in vitro* The

and also depress the blood lymphocyte count, but I find no record in the literature that this in fact happens (for example, in suicidal poisoning) We have not yet investigated the *in vivo* effects ourselves.

In connection with the effect of barbiturates on brain metabolism, search has been made for a biochemical lesion in the nerve cell It has been found in isolated mitochondrial preparations that 5×10^{-4} M Nembutal uncouples oxidative phosphorylation,[29] and that Amytal inhibits the oxidation of pyruvate and α ketoglutarate, but not that of succinate[30] In biochemical terms this points to a specific block in the oxidative pathway between flavin adenine dinucleotide (FAD) and cytochrome c In confirmation of this biochemical theory it has been claimed that intravenous succinate can rapidly reverse barbiturate poisoning in man[31, 32] We found, however, that 10^{-3} M succinate did not protect our lymphocyte cultures from the effects of Nembutal or Seconal So once again the lymphocyte seems to have its own private lesion

We remain unable to explain why lymphocytes are more sensitive than any other cells to the lethal action of ionizing radiation, cortisone, nitrogen mustard, colchicine, and barbiturates It may be noted, on the other hand, that lymphocytes are relatively resistant to environmental changes in tonicity, ionic balance, *p*H, CO_2, and to SH-poisons, urethane, and alcohol

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Colchicine. In the work now to be reported the toxicity of colchicine and various barbiturates was studied in the same way, except that a modified culture technique[15] and a synthetic medium[8] were used. We found that both colchicine and certain barbiturates, such as pentobarbital sodium (Nembutal), killed lymphocytes *in vitro* and that, like cortisone, their action was slow. The number of dead cells increased linearly with time over the first 48 hours (FIGURE 3). We therefore chose 48 hours as the end point and compared the effects of various concentrations of these substances (FIGURE 4).

Evidently colchicine is extremely toxic to lymphocytes—much more so than either cortisone or nitrogen mustard. A concentration of 3×10^{-8} M killed a measurable number of cells. As far as I know this is the lowest cytocidal concentration ever recorded for any chemical on any cell. If we assume a uniform concentration throughout cells and medium, it amounts to about 3000 molecules per cell. It is known that the administration of colchicine to the whole animal kills some of the lymph-node lymphocytes, but the effect has not been studied quantitatively. Apparently, an injected dose of about 2 mg./kg. in the rat has about the same effect as the same dose of nitrogen mustard.[22, 23]

The classic effect of colchicine, of course, is to cause metaphase arrest, with clumping of the chromosomes, in mitotic cells. It has been shown both with onion-root tips[24] and in fibroblast cultures[25] that this action of colchicine on mitotic cells can be completely antagonized by *meso*-inositol. We found, however, that 10^{-4} M *meso*-inositol did not alter the effect of colchicine on our lymphocytes. This, together with the fact that small lymphocytes rarely if ever divide, suggests that the lymphocyte-killing action of colchicine is unrelated, biochemically, to its metaphase-clumping action. There is a curious parallelism here to the effects of ionizing radiation, for the cells of the body most sensitive to radiation are the small lymphocytes, on the one hand, and mitotic cells on the other.

Barbiturates. The toxic effect of barbiturates on lymphocytes was first noticed by Lajtha,[26] who found that 10^{-3} M Nembutal killed most of the lymphocytes in cultures of bone marrow or leukemic blood without visibly affecting any of the other cells. We have confirmed this on our lymph-node cultures and have also investigated 4 other barbiturates, using the sodium salt in each case. In descending order of toxicity the series ran: secobarbital (Seconal), pentobarbital (Nembutal), amobarbital (Amytal), thiopental (Pentothal). Barbital (Barbitone) was entirely nontoxic over the range studied (FIGURE 4). This order bears no relation to their anesthetic potency, nor does it match any particular chemical configuration in the molecule. Pomerat and Painter[27] found that barbiturates were toxic to *in vitro* cultures of embryonic chick spinal cord. The concentrations required for complete suppression of outgrowth were: Seconal, 8×10^{-4} M, Nembutal, 1.4×10^{-3} M, and Amytal, 1.8×10^{-3} M (figures calculated from their data), which is in the same descending

than colchicine or cortisone,
kills no lymphocytes at $3 \times$
10^{-4} M. FIGURE 4 shows that there is a rather critical concentration, 10^{-4} M,

THE THYMIC LYMPHOCYTOSIS-STIMULATING FACTOR*

By Donald Metcalf

The Children's Cancer Research Foundation, Boston, Mass., and the Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

The functions of the lymphocyte in the normal animal are poorly understood. There is much evidence that the lymphocyte is a necessary part of the body defense mechanisms due to its role as a carrier of antibodies and by virtue of the accumulation of lymphocytes around inflammatory foci. In the normal animal there have been no clues to the reasons for the ubiquitous presence of the lymphocyte throughout the tissues of the body nor for the strict maintenance of circulating lymphocyte levels. These deficiencies in our knowledge have rendered difficult an intelligent approach to the problem of the maintenance of lymphocyte homeostasis.

It has long been known that extracts of the adrenal cortex produce a lymphopenia. There has previously been no evidence for circulating factors that might provoke a lymphocytosis in the normal animal. This paper describes the detection of a thymic lymphocytosis-stimulating factor and the interrelationships between this factor and other known influences on circulating lymphocyte levels. The role of this factor in the pathogenesis of lymphatic leukemia is also described.

Detection of the Lymphocytosis-Stimulating Factor (LSF)

The standard test animal used for demonstrating thymic LSF is the baby mouse. The thymus, mouse or human, is removed aseptically, and a saline suspension of thymus is prepared by grinding the thymus with penicillin saline (1000 units/ml.) in a pestle and mortar. The suspension is centrifuged at 3000 rpm for 15 minutes to remove cellular debris, and the supernatant fluid is collected. A single injection of 0.02 ml. of this supernatant fluid is made intracerebrally into each of 3 litters of mice (18 mice) aged 24 to 48 hours, and the injected mice are returned to their mothers. Six days later, white cell counts are performed on the tail blood of these mice, and the presence or absence of a lymphocytosis determined. Since there is considerable individual variation in the response to a standard stimulus, the mean lymphocyte level of the group is used to determine whether the inoculated fluid contains detectable LSF.

Effect of LSF in Baby Mice

Following the injection of active preparations of thymic LSF, a temporary lymphocytosis develops in inoculated mice. This lymphocytosis represents a 50 to 150 per cent increase in circulating lymphocytes, and reaches its maximum about 6 days following injection. The lymphocytosis passes off after 2 weeks (FIGURE 1).

No alterations in circulating polymorphonuclear leukocyte levels occur in in-

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mectomy leads to loss of detectable plasma LSF (in conditions where this is detectable),[9] and (5) localized irradiation of thymus leads to a fall in plasma LSF in the immediate postirradiation period.[8]

The thymic cell responsible for LSF production is not known. Anatomical dissection of the mouse thymus has shown[8] that the LSF is associated with the thymic medullary tissue, not the cortical lymphoid tissue.

In the mouse, the thymus medulla is composed of a variety of cells, between which is a plentiful eosinophilic ground substance. However, histological techniques used so far have failed to reveal which cell type is associated with LSF production. There has been some indication that the fluctuations in the amount of eosinophilic ground substance parallel fluctuations in LSF content, but this is as yet not firmly established.

It is felt that thymic LSF warrants classification as a true hormone on the following evidence:

(1) LSF is produced by a single ductless organ.

(2) It is carried in plasma but not in circulating cells.

(3) It acts at a distance on target cells. *The effect of LSF is not confined to an action on lymphocytic tissue in the neighboring thymus cortex. Injections of LSF into thymectomized mice are also effective in producing a lymphocytosis.*

(4) The *injection* of LSF produces a temporary effect not followed by permanent abnormalities. Subsequent injections of LSF are followed by lymphocytoses identical in character to that following the first injection.

(5) Circulating LSF controls the "feed-back" thymic production of LSF.

(6) LSF crosses species barriers.

Role of Thymic LSF in Lymphocyte Homeostasis

The observed circulating lymphocyte level in an animal may be considered to be the resultant of four factors: lymphocyte production, lymphocyte destruction or elimination, lymphocyte interchange between the circulation and the tissues, and the inherent natural life span of the lymphocyte.

Little is known of the pathways of normal lymphocyte destruction. It is likely that the spleen plays a prominent role, but the exact mechanism is unknown. *It is possible that adrenal corticoids may also regulate lymphocyte destruction.* Lymphocyte loss from mucosal surfaces occurs, but the magnitude of the process is disputed.[12]

Similarly, little is known concerning the mechanisms involved in the normal interchange of lymphocytes between the circulation and the tissues. Nothing is known of the factors determining the life span of the normal lymphocyte, but it is possible, as in the case of the red blood cell, that in some disease states the lymphocyte may be structurally different from the normal.

In this unsatisfactory situation, where three of the four factors influencing circulating lymphocyte levels are unknown, caution must be exercised in interpreting observations made on influences apparently regulating the rate of the fourth factor, lymphocyte production.

With these reservations in mind, some of the influences observed that ap-

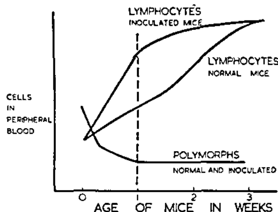


FIGURE 1 Lymphocyte and polymorph levels in the tail blood of baby mice injected with active preparations of thymic LSF

oculated mice This has made possible a more rapid assessment of the presence or absence of a lymphocytosis by calculating the lymphocyte polymorphonuclear leukocyte ratio from stained blood films

The lymphocytosis induced by thymic LSF rapidly reaches a maximum level with increasing concentrations of LSF After this point, further increases in the titer of inoculated LSF material produce no further elevation of the lymphocyte levels in the test mice This plateau response to LSF necessitates the use of serial dilutions of the thymic preparations when assessing the concentration of LSF in such material

No histological changes have been observed in the organs of injected mice, nor have any abnormal disease states been observed in injected mice observed for over 12 months following injection

The mechanism of the production of the lymphocytosis is unknown Three main possibilities exist (1) the release or redistribution of preformed lymphocytes, which is unlikely in view of the slow onset of the lymphocytosis, (2) an induced increased life span of lymphocytes, which is also unlikely but not impossible in view of the evidence[10] of increased life spans of lymphocytes in lymphatic leukemia where excess LSF levels have been shown to occur,[4] and (3) the increased production rate of lymphocytes by lymphopoietic tissues, for which, thus far, there is no direct supporting experimental evidence, but which has been accepted as the best working hypothesis

Nature of Thymic LSF

The evidence that the thymus is the only source of origin of LSF is as follows (1) the thymus is the only organ in humans and mice with detectable LSF except the plasma under certain circumstances,[5] (2) there is an increase in titer of LSF in thymic tissue cultures,[5] (3) thymic production of LSF influenced by the circulating level of LSF, and while the thymic LSF content in baby mice injected with LSF is below normal, LSF is rapidly reaccumulated when such thymuses are removed from the milieu of excess circulating LSF,[5] (4) thy-

Thymic LSF completes the triangle of basic forces influencing lymphocyte production. LSF exerts a stimulatory effect on the lymphopoietic cells, in magnitude somewhat less than the suppressive effect of the adrenals. As opposed to the 50 to 100 per cent rise in circulating lymphocytes in strain C57BL mice following adrenalectomy, thymectomy is followed by a gradual fall in lymphocyte levels. However, after several weeks, this fall is still only 30 to 40 per cent below base-line values [4]

Thymic LSF production is conditioned by the genetic make-up and the age of the animal. Thymic titers of LSF are higher in strains AKR and C58 than in strains C57BL and RF. In the mouse, thymic titers of LSF are very low at birth, rise progressively during early adult life and, in some strains, fall in old age

A second influence of the genetic make-up of the animal appears to be the determination of responsiveness or unresponsiveness of the lymphopoietic tissues to the stimulus of LSF.

From birth, mice of strains AKR and C58 show an apparent unresponsiveness to the stimulation of injected LSF This is seen as a failure to develop a lymphocytosis in baby mice or thymectomized adult mice when injected with [illegible]

lymphopoietic tissues [4]

Some types of trauma are capable of stimulating increased LSF output, resulting in a lymphocytosis [5] Thymectomy prevents this lymphocytosis response to trauma, but a lymphocytosis of the magnitude of that occurring in normal or sham-operated animals may be produced by the injection of cell-free LSF preparations Again, the genetic influence on responsiveness to LSF may be observed in the failure of strains AKR and C58 to develop this lymphocytosis response to trauma.[5]

There is evidence that the bone marrow may produce a substance regulating thymic regeneration following radiation injury [7]

In considering the role of thymic LSF in lymphocyte homeostasis in the mouse, two types of situation appear to exist

In the first, as exemplified by mice of strains C57BL and RF, relatively low resting lymphocyte levels are found, thymic LSF production is moderate, and such mice respond with a prompt lymphocytosis to injected LSF or following the stimulation of trauma In such mice, the maintenance of part of the circulating lymphocyte level appears to be dependent on thymic LSF

In the second type, as exemplified by strains AKR and C58, high resting lymphocyte levels are found, thymic LSF production is elevated, and there is an associated lack of responsiveness to LSF In such mice, the maintenance of circulating lymphocyte levels is independent of thymic function, and there appears to be a curious inflexibility of such lymphocyte levels when subjected to the stimulation of trauma

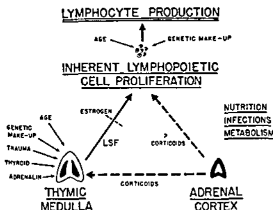


FIGURE 2 Schematic diagram showing the role of thymic LSF in the regulation of lymphocyte production in the mouse

parently affect lymphocyte production in the mouse are set out diagrammatically in FIGURE 2.

The greatest single force in lymphocyte production appears to be the inherent proliferative activity of the lymphocytic tissues themselves In the absence of known suppressing and stimulating influences, as in the adrenalectomized-thymectomized-splenectomized animal, high circulating lymphocyte levels are maintained [9] There is evidence that this inherent growth rate is in part genetically determined,[9] although external factors such as nutrition and general body metabolism are obviously potentially important The age of the animal may also directly influence the proliferative activity of the lymphopoietic cells [9]

The second major component is the constant suppression of lymphopoietic cell proliferation by the adrenal cortex This is considerable in the normal animal, and is increased in conditions of stress [8] The adrenal suppressive activity in the mouse appears to be divided equally between a suppression of the stimulating effect of thymic LSF and some extrathymic mechanism, possibly a direct suppressive effect on lymphopoietic cells [8]

Lymphocyte levels in two groups of young male C57BL mice were examined One group was thymectomized, the other sham-thymectomized One month later, both groups were subjected to bilateral adrenalectomy The resulting lymphocytosis was lower in the thymectomized group, but this difference could be corrected by the injection of cell-free thymic LSF preparations [8] This would indicate that part of the suppressive effect of the adrenal cortex on lymphocyte levels in the normal animal may be operative through thymic LSF In confirmation of this hypothesis, injections of cortisone in mice have been shown to reduce the thymic content of LSF [6] The genetic make-up of the animal may again regulate lymphocyte production via this adrenal suppression [1] Arnesen has produced evidence of strain differences in the adrenal cortex in mice [1] These may contribute to observed differences in lymphocyte homeostasis in various strains

meostasis, both by its direct stimulation of lymphopoietic cells and indirectly by its suppression by adrenal corticoids.

Overproduction of thymic LSF occurs in lymphatic leukemia in the mouse and man, and is intimately concerned in the pathogenesis of this type of leukemia.

Abnormal lymphocyte homeostasis in leukemic and preleukemic mice has been demonstrated

Acknowledgments

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Thymic LSF and Lymphatic Leukemia

Intelligent observations on the control of lymphocyte homeostasis are hampered by a lack of knowledge of the normal functions of the lymphocyte. However, evidence has been obtained that indicates that disorders of lymphocyte homeostasis exist in lymphatic leukemia and actually precede the development of the disease.

It has been found[4] that, in humans with chronic lymphatic leukemia and lymphosarcoma, the thymus contains greatly increased amounts of LSF. This is reflected in the plasma where, unlike the situation in the normal person, detectable amounts of LSF circulate.

In mice with lymphatic leukemia, a similar situation exists.

In the human, little can be done to determine whether LSF overactivity precedes or follows the development of leukemia However, an investigation of three cases of follicular lymphadenoma, a precursor disease state, also showed evidence of LSF overactivity

In the mouse, the use of high-leukemia strains of mice has enabled the position to be clarified

In the high-leukemia strains AKR and C58, evidence has been obtained that excess thymic LSF is present from birth and certainly precedes the leukemic state[7] As described above, an associated defect has also been revealed; namely, an unresponsiveness on the part of these mice to the stimulus of LSF. These abnormalities in preleukemic mice of strains AKR and C58 are accompanied by high resting lymphocyte levels and an inability to show a lymphocytosis response to trauma

It has been of considerable interest to find that low-leukemia strain C57BL mice, when subjected to leukemogenic doses of whole-body irradiation, develop all of these abnormal features

These changes appear to be of significance in the pathogenesis of genetically conditioned and irradiation-induced lymphatic leukemia It may be that the prolonged exposure of the lymphopoietic tissues to the stimulation of excessive levels of LSF, particularly under conditions where normal responsiveness to such stimulation is not possible, may result in the development of neoplasia in these tissues

Thymic LSF and Hodgkin's Disease

In four cases of Hodgkin's disease examined, no evidence of LSF overactivity was found This would suggest that Hodgkin's disease is fundamentally different from lymphatic leukemia and lymphosarcoma Further, if Hodgkin's disease represents a primary disease of the thymus as suggested by some,[11] such a tumor must be nonfunctional from the point of view of LSF production

Interestingly, patients with reticulum cell sarcomas also failed to show evidence of excessive LSF production

Summary

The thymus has been shown to produce a lymphocytosis-stimulating factor (LSF) This factor is involved in the maintenance of normal lymphocyte ho-

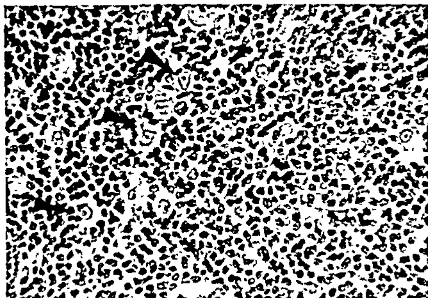


FIGURE 1. Pulp in hyperplastic lymph node. Interspersed among lymphocytes are "stem cells," indicated by arrows. These are characterized by large vesicular nuclei with prominent nucleoli. Cytoplasm is variable in amount and with poorly defined border. Paraffin section, hematoxylin and eosin stain. ×450.

disagreement as to their morphologic character. I must confess to a sense of tremulous intrepidity at being unimpressed with the totipotentiality of these elements. Other cells sparsely scattered in the pulp appear more likely to have hematopoietic capacities. These are considerably larger than lymphocytes possessing abundant, poorly defined cytoplasm that stains palely with acid dyes. Nuclei are large, spherical, sharply outlined, and contain scanty chromatin, but have characteristically prominent nucleoli (FIGURE 1). In order to assuage those easily ruffled, one would do well to designate these elements as "A" or "X" cells. On the other hand, since they are obviously primitive, have syncitial character, and are considered by many to constitute precursor elements, a more explicit name would seem desirable. It would meet favor with some to classify these cells as "reticulum cells" if this were truly a meaningful term. As I shall show later, this is not the case, and the term "stem cell" is proposed in view of the embryonal appearance of the cell and the cytological qualities which, in many respects, simulate the embryonic angioblast.

Also common is a cell that remotely resembles the stem cell and is occasionally mistaken for it. It, too, is large, but its cytoplasm is more clearly defined and frankly eosinophilic. Its nucleus is smaller, eccentric, and ranges from a spherical to a reniform configuration. Its chromatin, varied in character, only rarely exhibits a vesicular appearance. Nucleoli do not have the prominence seen in the more primitive elements and, of salient importance, phagocytic cyto-

Part II. The Reticulum Cell

THE CYTOLOGICAL IDENTITY AND INTERRELATION OF MESENCHYMAL CELLS OF LYMPHOID TISSUE

By Edward A Gall

Department of Pathology, College of Medicine, University of Cincinnati, and the Cincinnati General Hospital, Cincinnati, Ohio.

The title of this paper permits the assumption that the component elements of lymphoid tissue possess individuality and recognizable structural or functional characteristics A consideration of the interchanges that appear in publications on hematology and in monographs such as this introduces a note of doubt that complete accord exists Although disagreements are attributable in part to conceptual differences and in part to variations in technical methods, they also reflect impaired communication of a relatively high order. The latter is characterized by rigidities of nomenclature and by resistance to acceptance of any meaning save that individually determined Even truths topple when a keystone word finds disfavor or misinterpretation

A natural outcome of this situation is the impulse to embark upon personal enterprise This may conceivably lead to an extension of knowledge; instead, it usually results in an expansion of controversy. More often there is an effort at selective evaluation of existing views One may then conclude that all is confusion and that no stand is justified, or move to the other extreme and package multiple viewpoints into a hopeless jumble. Again, one may critically select a practicable composite and adhere to this with revision as newer data provide justification This last approach appears both flexible and reasonable, it is the one I propose to adopt

Fortunately, there is fairly general agreement upon the structural pattern of the lymph node and the related terminology Discretion would therefore dictate that discussion be instituted in this area The entrance and emergence of lymphatic channels and the circuitous seepage of their lymph content proceeding via the nodal sinuses from the convex surface to the concave hilar margin of the node are familiar features Similarly, the fibrous character of the capsule and trabeculae requires no elaboration To these are anchored the delicate argyrophilic fibrils of reticulin, the derivation of which has been the subject of debate for years[1, 2] The advent of refined methods of study, in-

y by indicating the

, it is probably the

contiguity or dis-

persion Of course, it may be argued with equal validity that the degree of cellularity itself determines the nature of the net Adjacent to the capsule and trabeculae, the sinuses contain only sparse and loosely hung fibrils that join the denser network of the pulp at the sinus margins The pulp network is finely and rather uniformly meshed, but again becomes scanty and poorly defined in the cortical compartments in relation to the lymphoid follicles

Lymphocytes comprise the bulk of nodal cellular content There is little

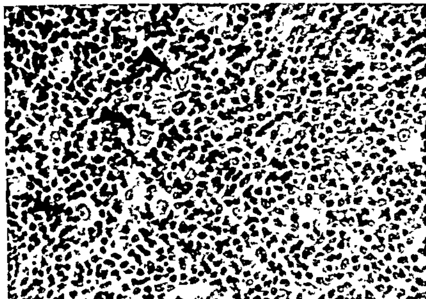


FIGURE 1. Pulp in hyperplastic lymph node. Interspersed among lymphocytes are "stem cells," indicated by arrows. These are characterized by large vesicular nuclei with prominent nucleoli. Cytoplasm is variable in amount and with poorly defined border. Paraffin section, hematoxylin and eosin stain. ×450.

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FIGURE 2 Sinus in hyperplastic lymph node The sinus contains packed "histiocytes" Nuclei resemble those of stem cells, but are smaller and more irregular in configuration Chromatin is more finely dispersed, and nucleoli are not prominent
Paraffin section, hematoxylin and eosin stain ×450

plasmic qualities are often apparent This cell appears in the pulp, in active lymphoid follicles and, in varying abundance, in the sinuses (FIGURE 2) By reason of its morphologic and functional characteristics, the term "histiocyte" appears applicable I shall not insist on this name since, as indicated in TABLE 1, there is a wide choice of alternatives.

TABLE 1
NOMENCLATURE FOR THE HISTIOCYTE ENCOUNTERED IN THE LITERATURE*

Histocyte	Monocyte
Macrophage	Histogenic monocyte
Clasmatocyte	Large mononuclear
Polyblast	Large mononuclear lymphendothelіocyte
Pericyte	Lymphendothelіocyte
Perithelial cell	Hemendothelіocyte
Adventitial cell	Reticuloendothelіocyte
Anode cell	Reticuloendothelial cell
Rhagiocrine cell	Endothelial cell
Pyrrhol cell	Endothelial leukocyte
Dictocyte	Endothelioid cell
Interstitial cell	Littoral cell
Transitional cell	Kupffer cell
Resting wandering cell	Microglia

*Reproduced by permission of the American Society of Clinical Pathologists [10]

In addition to each of these clearly defined prototypes there are intermediary forms representing phases of transition. Unfortunately, there are no signposts [illegible]

in hematopoiesis is a moot point.

The variety and relative proportions of cells in lymphoid tissue are indicative of the susceptibility of this organ to provocations of a metabolic, inflammatory, or neoplastic nature. There is little quiescence in lymph nodes which, indeed, are in a state of constant histological motion, nor is there reason to believe that the pattern in one node necessarily reflects that in another. In the same patient one may find pulp hyperplasia, sinus hyperplasia, or follicu-

TABLE 2
ENZYME ACTIVITY IN LYMPH NODE[3]

	Lymphocyte	Histiocyte	Stem cell	Endothelium
Esterase Acid phosphatase Phosphamidase	0	+	0	0
5 Nucleotidase	0	0	±	0
Alkaline phosphatase	0	0	0	+

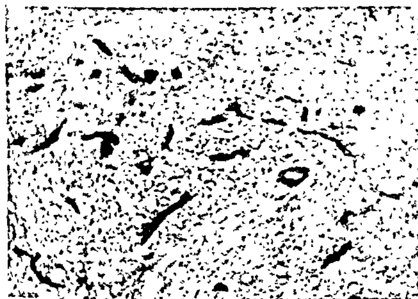
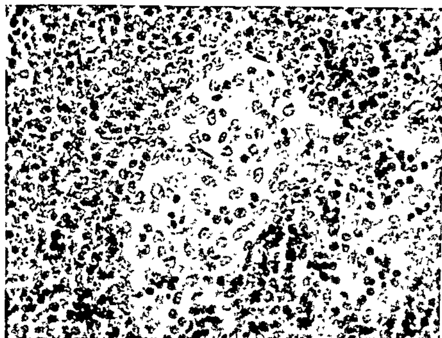


FIGURE 3. Acetone fixed normal lymph node, paraffin embedded. Stained by the diazo technique with sodium alpha naphthyl acid phosphate to demonstrate alkaline phosphatase activity.[3] This is characterized by dark staining and appears only in vascular channels lined by endothelium. ×15



[illegible] ins packed "histiocytes"
[illegible] rregular in configuration

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Resting wandering cell	Microglia

* Reproduced by permission of the American Society of Clinical Pathologists [10]

in the lining endothelium of small lymphatics and vascular channels (FIGURE 3). Histiocytes, on the other hand, contained a high level of acid phosphatase, esterase, and phosphamidase. This was the case, whatever the location of the cell in the pulp, follicle, or sinus (FIGURE 4). Of interest is the fact that this type of staining disclosed a cellular configuration identical with that demonstrated by the silver-deposit methods[1] and quite unlike that encountered with hematoxylin and eosin staining in paraffin-embedded tissues (FIGURE 5). Similar staining was not encountered in stem cells that exhibited minor but significant reactivity only with the 5-nucleotidase method

Lymph node cells, therefore, are distinguishable on both a morphologic and a functional basis The application of similar histochemical methods to diseased nodes reveals retention of identical histochemical features This was the case in instances of sinus hyperplasia, tuberculosis, and sarcoid where histiocytes could be regularly highlighted. In carrying the studies further in examples of malignant lymphoma those characterized by the presence of histiocytes continued to exhibit specific reactivity (FIGURE 6) Other forms of lymphoma in which lymphoid elements predominated were devoid of staining as in their nonneoplastic counterparts. In a single specimen of primitive (stem cell) lymphoma, only 5-nucleotidase activity was manifest, and this in small amounts.

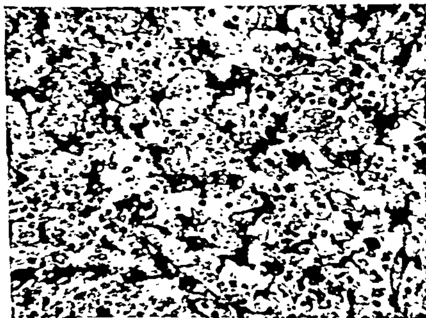


FIGURE 5 Section from node similar to that shown in FIGURE 4 Frozen section impregnated with silver carbonate[4] to highlight histiocytes These cells show stellate configuration as in FIGURE 4 In addition, elongated delicate fibrillar intercellular connections may be seen ×900

lar hyperplasia in different nodes simultaneously. There is no clear knowledge as to which stimulus specifically induces a given pattern.

In an effort to define the functional qualities of lymphoid elements more closely, a host of new technical procedures have been developed in recent years This represents a resurgence of a methodology that had a transitory popularity at the turn of the century and for about twenty years thereafter. There is neither the space nor the necessity to describe here the results of these advances, many of which are presented elsewhere in this monograph. However, I shall report to you succinctly some studies now in process in our own laboratories.[6, 7] Although incomplete at the moment, they are nevertheless of interest at this point.

In brief, we have applied certain histochemical techniques to lymph nodes with the purpose of determining the various forms of enzyme activities in different cellular components Of particular note at the moment are the results obtained with a limited battery of procedures for the detection of alkaline and acid phosphatases, nonspecific esterase, phosphamidase, and 5-nucleotidase. In each instance several methods have been used and, under controlled conditions, they have been found to be reasonably exact and reproducible. The results, though consistent in our hands, differ in some details from those described by others This is probably attributable to certain refinements of technique As indicated in TABLE 2, lymphocytes failed to show any evidence of activity of the five enzymes sought Alkaline phosphatase appeared only

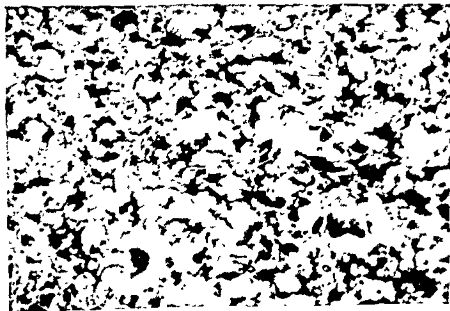


FIGURE 4 A lymph node the seat of reactive histiocytosis to demonstrate nonspecific esterase activity in the histiocytes Frozen dried material stained with the naphthol AS acetate technique[7] Histiocytes are dark stained and have an irregular stellate configuration ×900

in the lining endothelium of small lymphatics and vascular channels (FIGURE 3). Histiocytes, on the other hand, contained a high level of acid phosphatase, esterase, and phosphamidase. This was the case, whatever the location of the [illegible], the fact that this [illegible] with that demon- [illegible] encountered with hematoxylin and eosin staining in paraffin-embedded tissues (FIGURE 5). Similar staining was not encountered in stem cells that exhibited minor but significant reactivity only with the 5-nucleotidase method.

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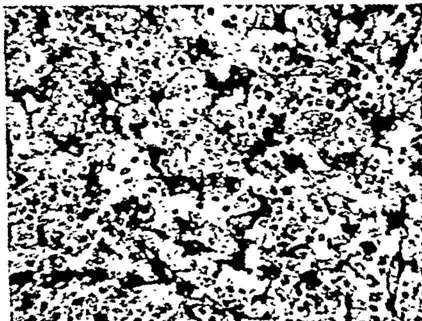
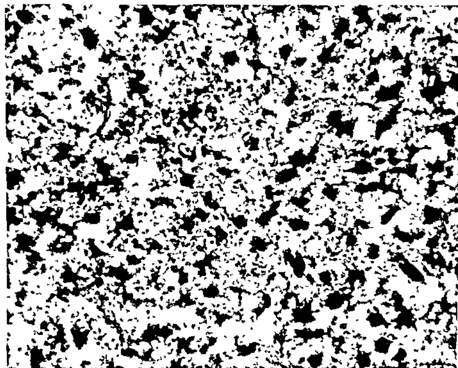


FIGURE 5 Section from node similar to that shown in FIGURE 4 Frozen section impregnated with silver carbonate[4] to highlight histiocytes. These cells show stellate configuration as in FIGURE 4 In addition, elongated delicate fibrillar intercellular connections may be seen ×900



[illegible]

There has been a studied effort in this discussion to avoid the utilization of the expressions "reticulum cell" and "reticulum cell sarcoma." Such an avoidance confessedly borders upon stubborness, for there are few terms with wider usage among hematologists. Unfortunately, a probe into meaning leads to a chaotic miasma.[9] Even conceptual views are at wide variance, as indicated by TABLE 3. Moreover, a compilation of the morphologic criteria that have been cited in delineation of the reticulum cell is no less confusing. Most authors simply assume that the reticulum cell has universal recognition and

TABLE 3

FUNCTIONAL CONCEPTS OF THE RETICULUM CELL*

(1) Primitive totipotential hematopoietic cell
(2) Hematopoietic and stroma-forming cell
(3) Phagocytic cell and/or precursor
(4) Argentophil fibril forming cell
(5) Vascular (endothelium-like) lining cell
(6) Any 2 or more of above

* Reproduced by permission of *Minnesota Medicine*.[9]

TABLE 4
HISTOLOGICAL CHARACTERISTICS OF RETICULUM CELL SARCOMA AS NOTED IN THE LITERATURE*

Size of Reticulum Cell		*Giant Cells*	
Large	9	Frequent	5
Small	8	Rare	4
12 to 15 μ	3	Absent	3
15 to 22 μ	3	Resemble Reed cells	5
22 to 50 μ	4	Do not resemble Reed cells	4
Configuration of Reticulum Cell		*Cytoplasm of Reticulum Cell*	
Syncytial	20	Abundant	14
Pseudopodal	10	Scant	13
Ill defined	7	Clear	6
Polygonal	4	Granular	5
Stellate	3	Basophilic	6
Round or oval	6	Eosinophilic	14
Fusiform	3	Neutrophilic or amphophilic	8
Varied combinations	14	Phagocytic	9
		Nonphagocytic	5

Nuclear Characteristics

Large	6	Round or oval	14	Vesicular	27
Small	5	Reniform	9	Hyperchromatic	5
Varied	4	Lobulated, irregular	9	Pale or even staining	7
2 to 7 μ	3	Mitosis, frequent	10	Membrane distinct	10
7 to 10 μ	3	Mitosis, rare	3	Membrane thick	6
10 to 12 μ	2	Mitosis, absent	3	Membrane thin	3
				Membrane folded	2

Nucleolus

Prominent	20
Not prominent	9
Absent	4

* Reproduced by permission of *Minnesota Medicine*.[9]

make no effort to describe it. Among those who do define its qualities, the characteristic features as listed by some have no resemblance to those described by others (TABLE 4). The reticulum cell appears to be a myth, and the retention of the term would seem to serve no useful purpose (FIGURE 7). Nevertheless, I am certain that no one who now utilizes the expression will hesitate to continue doing so.

Can one truly classify the forms of malignant lymphoma and not have at hand "reticu[illegible] debate and r[illegible] Hematology [illegible] and I presented a seminar on lymph node diseases in New Orleans, La., under the sponsorship of the American Society of Clinical Pathologists.[10] What began as an apparently irreconcilable divergence of views under the pressure of a need to synthesize a workable classification ended in harmonious agreement. The classification we ultimately presented had the value of comparative simplicity, a susceptibility to illustration and ready discrimination, an applicability to clinical phenomena and, of some significance, an indication that minds in opposition could meet in compromise (TABLE 5).

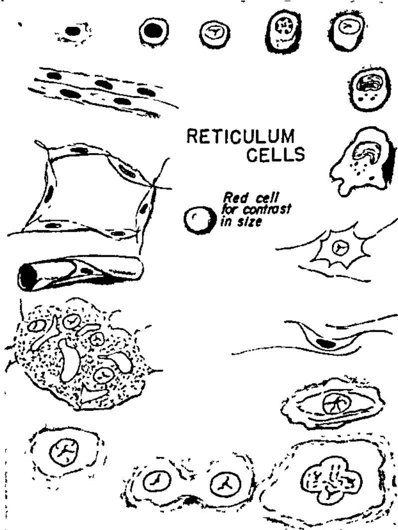


FIGURE 7 Sketch illustrating the construction of the "reticulum cell" in accordance with various descriptions encountered in the literature Reproduced by permission of *Minnesota Medicine*.[9]

For many years students of lymphoma have believed that a pathogenetic [illegible] This has been [illegible] ant lymphoma" [illegible] been supported by the recognition that metamorphosis from one form of lymphoma to another could be anticipated in a certain proportion of cases.[11-13] The occurrence of variation and dedifferentiation has been likened to similar alterations appearing in epithelial neoplasms. How broad this variability of pattern may be in

Table 5

Working Classification of Malignant Lymphoma*

"Nodular" (Follicular) Diffuse

(1) *Stem cell* (2) *Histiocytic*	"Reticulum cell sarcoma"
(3) *Lymphocytic*	
(a) Poorly differentiated (b) Well differentiated	"Lymphosarcoma"
(4) *Mixed cell*	(Histiocytic and lymphocytic)
(5) *Hodgkin's*	
(a) Paragranuloma	
(b) Granuloma	("Classic")
(c) Sarcoma	

* Reproduced by permission of the American Society of Clinical Pathologists [10]

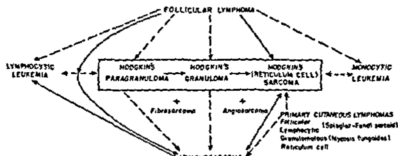


Figure 8. Transitions observed among types of lymphoma [11] Reproduced by permission of *Radiology*.

lymphoma is indicated by the vector chart devised by Custer[11] (figure 8), which would indicate a potentiality for extensive histological flux. Indeed, Custer has stated that in his experience such variations may be anticipated in due course in 40 per cent of cases. Some years ago, in an effort to quantitate this phenomenon, B Castleman and I investigated a series of cases in which serial biopsy specimens or specimens procured at both biopsy and necropsy were available (unpublished data) In an effort to provide sufficient interval for transformation to occur, we selected cases in which at least 3 months intervened between specimens This analysis concerned approximately 65 cases, and it was noted, as anticipated, that 35 per cent exhibited a change of lesion from one type of lymphoma to another In the course of deliberation on these results it seemed that the interval selected was an arbitrary one, accordingly, specimens were examined from all cases with more than one microscopic section available regardless of the dates of procurement To our surprise the same alteration rate of 35 per cent also occurred here In some of these the interval between excisions was one week or less, and it seemed unlikely that transformation could occur in so brief a period Thereafter, blind studies were made of a series of well-preserved sections obtained at necropsy from different lymph nodes in the same patients with malignant

lymphoma; again a significant variation of pattern was encountered. This was ordinarily of the nature of variations in degrees of differentiation, but on
lude
the
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malignant lymphoma, whatever its nature, possesses the capacity to induce different morphologic responses in the same person. The concept that lymphoma may alter its histological nature over the course of time requires re-evaluation. Naturally, one may argue that lesions may differ in duration in a given individual and that differences in pattern may indeed represent transformations in the course of development

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ASPECTS OF THE RETICULOENDOTHELIAL SYSTEM STUDIED WITH THE LIGHT MICROSCOPE AND THE ELECTRON MICROSCOPE *

By Leon Weiss †
Department of Anatomy, Harvard Medical School, Boston, Mass

The reticuloendothelial system, first clearly demonstrated by Goldmann (1909), is a system of connective tissue cells through which are expressed whatever potentialities for cellular differentiation persist from mesenchyme It has been recognized as comprised of cells concentrated in hematopoietic and endocrine tissues and fixed to a fibrous extracellular reticulum, and of cells free of reticulum and dispersed through almost every connective tissue (Maximow, 1924)

The reticular cell is the preponderant type among the fixed cells Flattened as endothelium, it may make up the walls of sinuses or, in stellate form, may constitute less well-organized parts of reticular tissue. In either case the reticular cell remains a multipotent connective tissue cell capable of differentiating into any of the blood cells, into macrophages, fibroblasts, and fat cells and, possibly, into other connective tissue cell types. Cytologically, reticular cells are undifferentiated, having large spherical nuclei and pale extensive cytoplasm They lack even the compacted basophilic cytoplasm, conspicuous nucleoli, and prominent nuclear membranes that signify the synthesis of protein and that, in fact, constitute in primitive blood cells the first evidence of differentiation from reticular cells Fixed cells may become free of the reticulum Such detachment may actually represent cellular maturation and may restrict the subsequent capacities of these cells for differentiation Indeed, the capacities for transformation of the free cells of the reticuloendothelial system have been the source of extended controversy Histiocytes (and monocytes that may evolve into histiocytes) are clearly phagocytic and may elaborate adaptive enzymes There is, moreover, increasing acceptance of the unitarian view that these cells and lymphocytes are but minor variants of the same multipotential cell type

In this presentation I shall consider certain aspects of the reticuloendothelial system that have particular reference to this monograph, and not attempt a systematic treatment I propose to discuss the sinuses in the red pulp of the spleen of rats, the relationship of splenic cords to splenic sinuses, the nature of the reticulum, a cytoplasmic inclusion induced in reticuloendothelial cells of guinea pigs by estrogens, the Foà-Kurloff body and, finally, techniques for the study of the dermal bone of the nine-banded armadillo. My discussion will be primarily morphologic, based upon observations with both the light and the electron microscopes

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In a recently published study of the red pulp of the spleen in human beings and rats (Weiss, 1957), splenic cords were interpreted as collapsed splenic sinuses lying between patent sinuses. The endothelium of splenic sinuses has a markedly irregular luminal surface. In a collapsed vessel, its endothelium crumpled, the lumen persists only as an uneven slit, and the vascular nature of the structure is often obscured. It had, in fact, been recognized, even before the classic work of Mollier in 1911, that the preponderant cell type in the cords is the same as that making sinal walls, and that sinuses merge indistinguishably into cords. The walls of sinuses, moreover, are common to two or more vessels, the repeating structure of the red pulp being lumen, endothelium, reticulum, endothelium, lumen, endothelium, reticulum, endothelium, and so on. Apertures are present in the walls of sinuses, but they constitute simply anastomotic passages and not communications to extravascular tissue.

The red pulp of the spleen would then be represented as an enormous vascular space made up of sinuses sharing common walls, communicating through *anastomotic apertures, having highly irregular contours, and following a* tortuous course Some sinuses are patent and some are collapsed Collapsed vessels lying between patent vessels constitute Billroth or splenic cords It is within such collapsed sinuses that phagocytosis and cytopoiesis appear most active If this conclusion is correct, it should be possible to convert splenic cords to splenic sinuses and sinuses to cords. In experiments designed to convert cords to sinuses, the spleens of rats were dilated by perfusion, passive congestion, and the administration of acetylcholine and of nitrites in eserinized rats Moreover, since the greater part of phagocytosis and cytopoiesis occurs in the cords, attempts to convert sinuses to cords were made by provoking phago-

[illegible]

expanded by blood In the adult, these places differed from unmistakable sinuses in one major respect, their walls were made up of cells filled with phagocytized material Many of these cells, particularly those almost completely filled with ingested material, were removed from the wall, in contact only by tenuous cytoplasmic connections Many others maintained an endothelial location although they were considerably loaded down, and often some cells with little or no phagocytized material were [illegible] distinguishable from unaltered sinal endothelium (FIGURE 1) [illegible] in the cord tissue or, as I should interp[illegible] collapsed vessels in which the flow of blood is stilled A cell filled with ingested material appears to abandon its role in making a wall This phenomenon may be accentuated in experiments forcing phagocytosis upon an endothelium until the morphology of few vessels is left undisturbed Often, where a cell becomes engorged with material and leaves the wall, the reticulum that presumably [illegible] would seem to

[illegible] eatures of note

It is a granular material containing no discernible cross-banded reticular fibers Reticular fibers may exist, of course, masked by ground substance Arranged

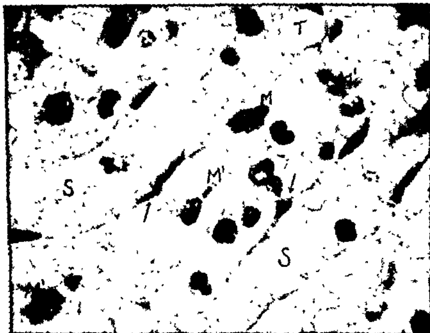


FIGURE 1. Light micrograph of the spleen of a rat. The spleen of an adult rat was distended by ligating the splenic vein. The vascular pedicle was then tied off and the entire organ removed and fixed in its distended state without loss of blood. (I am indebted to William Bloom for suggesting this procedure.)

Two sinuses (S) run obliquely across the field, their endothelial cells flattened by high venous pressure. Erythrocytes are unstained. Between these vessels is tissue that presumably had been cord tissue, but is considered to represent a sinus. In two places, portions of endothelial cell nuclei face on the lumen of this sinus (arrows). In other places cells in endothelial position have ingested material and largely come off the wall (M). Such cells occasionally appear to be isolated in the center of a sinus (M'). By serial sectioning it can be determined that these cells, too, occupy endothelial positions above or below the plane of section. At T a branch of this sinus comes off at a right angle and may be seen in cross section. The tissue in the upper left and lower right areas in the field may be interpreted in the same way.

The tissue was embedded in paraffin and stained with the periodic acid Schiff method and hematoxylin. ×1300.

in the pattern it normally takes, the reticulum appears fibrous only in the sense that a line of chalk or a trail of sand does so. In many places, moreover, the reticulum of a sinus is exposed directly to the circulation and, occasionally, the substance of the reticulum appears to be washed into the circulation. In periodic-acid-Schiff-stained preparations, one can often see a magenta wash in the lumen of sinuses, representing the substance of reticulum carried from its linear localization. A substance that may be identical to the substance of reticulum may be present in the basal cytoplasm of the endothelium and, in not a few instances, morphologic continuity between intracellular and extracellular substance may be observed. These observations invite certain inferences

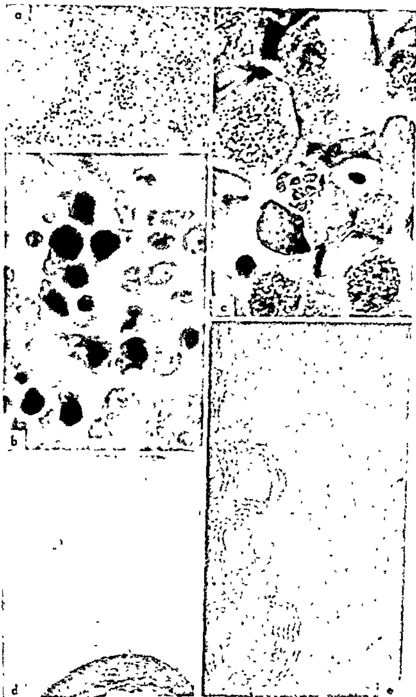


FIGURE 2 Foà Kurloff cells in guinea pig spleen (*a*) Light micrograph Foà Kurloff cells, selectively stained, are present in red pulp, concentrated, in some cases, in the marginal

The reticulum would appear to have functions in addition to, and perhaps more important than, the intuitively accepted function of support for the sinal wall. The presence of material identical to reticulum in the endothelial cytoplasm suggests that reticulum is secreted by the endothelium that lies upon it. The absence of reticulum in places where endothelium has left the wall suggests that there is a significant turnover in reticulum and that the endothelium is necessary for its renewal.

If the red pulp is a vascular space, it is clearly important to learn whether or not there exists a mechanism that determines where blood flows within its sinusal system beyond the point of arterial control. I suggest that the reticulum may represent an unstable grid upon which the endothelium is aligned and through which the direction of blood flow may be controlled. In a system of vessels having common walls, the movement of one part of the wall could open one vessel and close another In argyric rats the uptake of silver is most pronounced in the reticulum of the sinuses of the marginal zone, that specialized part of the spleen that constitutes a transitional tissue between white pulp and red pulp (Snook, 1950). Here the sinuses are large and arranged more or less circumferentially around the white pulp. The sinuses of [illegible] Those [illegible] In [illegible] ginal zone are first filled Perhaps it is through changes in the walls of the marginal zone, from the outer aspects of these circumferential vessels, that the flow of blood into the deeper vessels of the red pulp is controlled as the endothelium and the reticulum make new alignments. Further in the red pulp similar adaptations may occur

I now turn to a related, but specialized, reaction of the reticuloendothelial system, the Foà-Kurloff cell. Foà-Kurloff cells are cells of the reticuloendothelial system of guinea pigs and are identified by cytoplasmic inclusions and elicited by estrogenic stimulation (Ledingham, 1940). I became interested in these cells because of their presence in the red pulp of the spleen, the histo-



FIGURE 2 Foà-Kurloff cells in guinea pig spleen (*a*) Light micrograph Foà Kurloff cells, selectively stained, are present in red pulp, concentrated, in some cases, in the marginal

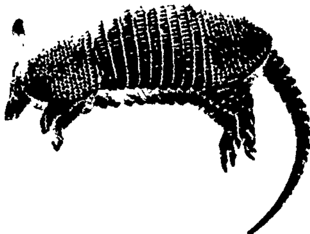




FIGURE 3 (a) A nine-banded armadillo ... the anterior shield

This animal has been ...

chemical similarity of their inclusions to reticulum, and their evocation by estrogen The effects of estrogen upon ground substance are known, and I thought that perhaps the Foà-Kurloff cells actually might be producing reticulum under estrogenic stimulation. At present this seems unlikely, but I believe my preliminary findings are of sufficient interest to present here

In tissue sections the inclusions are of a homogenous, presumably liquid, material uniformly stained with the periodic-acid-Schiff technique. They have been characterized histochemically as a mucoprotein (Pearse, 1949). In smear preparations of blood, spleen, and bone marrow, the inclusions tend to be particulate and basophilic (FIGURE 2, *a* to *d*). The inclusions may be multiple, barely visible structures indistinguishable in the light microscope from granules commonly present in lymphocytes, or single and large enough to fill a lymphocyte Normally representing less than 1 per cent of guinea pig blood cells, Foà-Kurloff cells may rise to more than 30 per cent of blood cells and may comprise a great portion of the cells of the red pulp of the spleen, bone marrow, and thymus (Simmons, 1956) The volume of the spleen may be increased twofold by the accumulation of these cells (Nadel, 1952). Lymphatic nodules contain few Foà-Kurloff cells, a finding that suggests a basis for distinguishing mononuclear blood cells originating in lymphatic nodules from those originating elsewhere.

In the electron microscope the inclusion consists of an apparently liquid phase, presumably the mucoprotein, and a peripherally placed particulate phase, presumably the basophilic component The particles measure up to 0 4μ in diameter and are made of highly ordered lamellated membranes, often forming a more or less spherical structure and enclosing a fine granular core. The liquid phase has a faint lamellar character. Surrounding the particulate component, these lamellae in the liquid component are relatively pronounced and actually become continuous with those of the particles Therefore, fluid and particulate components appear to be phases of the same system (FIGURE 2, *d* and *e*) The significance of this remarkable inclusion is not yet known

Finally, I refer to the dermal bone marrow of the nine-banded armadillo, *Dasypus novemcinctus*, to present methods for its study In collaboration with the late George B Wislocki (Weiss and Wislocki, 1956), seasonal variations in hematopoietic activity were observed and taken to represent the influence of environmental temperature This marrow passes through a phase that consists almost entirely of reticular cells This tissue, located superficially and distributed to more than 600 locules, is suited to experimental alteration It should be possible to determine the effect of temperature on hematopoieses by maintaining an armadillo in a cold room with one half its carapace kept warm by a heating jacket In collaboration with H J Wheelwright of The Army Chemical Center, Edgewood, Md, moreover, the techniques for serial biopsy and roentgenography of the eaves of the carapace have been developed (FIGURE 3) The dermal marrow of the armadillo, simplified in its transitional stages and, presumably, experimentally influenced by temperature, would represent an organ in which the organization and responsiveness of the reticuloendothelial system could be determined

SOME *IN VITRO* AND *IN VIVO* STUDIES ON SEVERAL MESENCHYMAL CELL TYPES BEARING ON THE PROBLEM OF THE RETICULOENDOTHELIAL SYSTEM*

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Introduction

Just what the reticuloendothelial system (RES) is appears to depend largely upon how it is defined (Heller, 1957) To some, the histiocyte is the cell of prime concern, as phagocytosis of some kind is the only clearly established functional attribute of this system (Policard, 1957) To others, the RES is a particular physiological or functional cell state, "the histiocytary state" (Chevremont, 1942, 1943, 1945, 1948, 1955), which is completely independent of genetic limitations and can be achieved by many diverse types of cells. The acquisition by specific cells of some of the morphologic and phagocytic functional characteristics of the histiocyte has been viewed as a cellular "modulation" (Weiss, 1944, 1949).

In addition to the more conventionally designated RE cells, such as the fixed and free histiocytes (variously known), the reticulum cells, the lining cells of lymph and certain vascular sinuses, and monocytes (Bruman, 1935, Doan, 1957; Rebucl [illegible]
cells (Weiss, 1944, [illegible]
mont, 1943, 1955), [illegible]
[illegible] 57), germinal and mesothelial
[illegible] lymph and blood vascular
[illegible] lischul, 1954), ordinary connective tissue fibroblasts (Bruman, 1935, Chevremont, 1955, Policard, 1957), and fat cells (Burkhardt, 1934), as well as others, have all been reported either (1) to have the capacity to segregate within their cytoplasm colloidal particles and/or cell fragments or (2) to have the capacity to transform into or give rise to histiocytic type cells Our current dilemma relative to the RES and the several criteria used in its identification and in evaluation of its functional level was clearly envisaged thirty years ago by Maximow (1928, page 429, paragraph 3, line 7) "As the cells just enumerated include not only elements of mesodermal origin, but also of entodermal (thymic) and ectodermal (glial) origin, the outlines of the conception of the histiocytes in the case of such an extension are liable to become vague The storing and intraplasmatic transformation of the substances mentioned above [dyes, fats and endogenous pigments] at least as a temporary or pathological phenomenon, seem not to be the exclusive prerogative of the histiocytes "

* The [illegible] 49 of the Helen [illegible] of the Committee [illegible]
[illegible] Public Health Se[illegible]
of Oklahoma Me[illegible]

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detail can be discerned, and second, large (25 μ), slightly stellate cells having abundant homogeneous to slightly vacuolated cytoplasm and a relatively pale, vesicular nucleus with one or two nucleoli An occasional multinucleate cell is present (FIGURES 1 and 2) A basement membrane of sorts is present at the several junctions of the extraembryonic mesoblast with the epithelia of the entodermal, amniotic, and trophoblastic vesicles (FIGURE 2) The large cell type closely resembles the "embryonic-type" fibroblasts of the predecidual and early endometrial lamina propria (compare FIGURE 2 with FIGURE 11).

cell population other than circulating blood cells during the early embryonic period

The mesoblast of a 14-day human ovum. In this ovum, the extraembryonic mesoblast has been incompletely cleaved by the formation of the extraembryonic coelom so that the amniotic, entodermal, and trophoblastic vesicles have acquired an extraembryonic layer of mesoblast of variable thickness

two types of

ajor axes and

of granular,

homogeneous, or vacuolar character. Its nucleus is large and vesicular with one or two prominent nucleoli and may be pleomorphic (FIGURE 7). The second cell type is small (15 to 20 μ) of irregular distribution and sometimes grouped (FIGURE 7) Its nucleus is small and more hyperchromatic than that of the larger cell type. The small cell type may be equivalent to the angioblast (Hertig, 1935) There are no discernible blood islands or vessels present anywhere in the extraembryonic area The large cell type resembles closely the reticulum cell of adult lymphoid tissue (compare FIGURE 7 with FIGURES 32 and 33)

The embryonic area, which in the 10- to 11-day ovum consisted only of contiguous portions of the amniotic and entodermal vesicles, shows the entodermal and amniotic ectodermal layers to be separated by a layer of loosely

migrated bodily out of the primitive streak, as some are in mitosis (FIGURE 4) The embryonic mesoblast is composed descriptively of two cell types The first is moderately stellate (12 to 18 μ) with variably vacuolated cytoplasm and coarse processes Its nucleus is large and hyperchromatic with one or two prominent nucleoli (FIGURE 4). The second cell type is small (8 to 10 μ), with intensely basophilic cytoplasm and of rounded to slightly stellate form Its

cells are morphologically dissimilar to the extraembryonic mesoblast cells of this ovum (FIGURES 3, 4, and 7). The entire substance of the embryonal

It is generally agreed that the reticulum cell of orthodox lymphoid parenchyma is endowed with pluripotential capacities. However, it has been stated that many of the borderline RES cells are also pluripotential. Thus it has been reported, as with other cell types, that vascular endothelial cells may transform into fibroblasts (Maximow, 1928, Altschul, 1954) or monocytes (Doan, 1957; Toro, 1947), and that fibroblasts may transform into mesothelial cells (Clarke, 1915–1916, Lewis, 1923a, 1923b) and into histiocytes and are really only slightly differentiated mesenchymal cells (Policard, 1957). The inherent possibility of error in evaluating such cellular transformations *in vitro* was clearly recognized by Bloom (1937), who pointed out that it is extremely difficult to distinguish between genetically limited cellular differentiations and cellular modulations as reflected by alterations in cell morphology and function.

In view of the present ambiguous genetic and morphologic status of the RES, it has seemed desirable to refocus our perspective of the entire problem: first, by a consideration of the early development of the human ovum, with special reference to the developmental basis for the anatomic, genetic, and functional correlations between certain mesodermal cell populations that are intimately interrelated to each other and to several facets of the RES problem and, second, by a consideration of the parameters of some of the inflammatory responses of several RES-implicated cells and tissues *in vitro*

Embryonic and Extraembryonic Mesoblast of Two Early Human Ova and Embryos, with Special Reference to the Peritoneal (Coelomic) Epithelial Cell Line Versus the Diffuse Mesenchymal Cell Line

Precise genetic and functional interrelations are not clearly evident for all RES-implicated cells in all locales of the body. However, as is well shown by embryologic literature (Patten, 1947, Arey, 1954, Hamilton, Boyd, and Mossman, 1945; Richter, 1953, Holyoke, 1936, Thiel and Downey, 1921, and others), there are several fully differentiated cell species, of variably close functional and anatomical importance to the RES problem, which can be traced in clear-cut histological continuity from the very earliest generations of embryonic mesoblast cells They are (1) the reticulum cell population of the spleen, (2) the cortical epithelial cell population of the adrenals, (3) the several physiological parenchymal cell types of the gonads, including the interstitial, granulosum, and germinal epithelial cells (Goldsmith, 1932), and (4) the parenchymal lining epithelium of the oviducts and uterus

The mesoblast of a 10- to 11-day human ovum This ovum (Richter, 1953) is comprised (1) of a blastocyst or trophoblastic vesicle filled loosely (2) with extraembryonic mesoblast (derived heteroplastically from the trophoblastic epithelium) that completely invests the (3) inner cell mass derivatives the amniotic vesicle and entodermal vesicle (FIGURE 1) No embryonic mesoblast is present in the embryonic disc proper (that is, the zone of contiguity between the amniotic and entodermal vesicles) No primitive streak is present Descriptively, the extraembryonic mesoblast (FIGURES 1 and 2) consists of two cell types: first, small (9 to 10 μ), spindle-shaped to round cells with slightly basophilic cytoplasm and an intensely hyperchromatic nucleus in which little

designated mesenchymal cells (FIGURE 9), the latter must be a differential step up in the line. The term mesenchymal cell is too frequently used to include all cell types of mesoblast or mesodermal origin (Aegerter and Long, 1949).

In marked contrast to the diffuse, obscure developmental history of the cells of the migratory or mesenchymal line is that of the peritoneal epithelial line whose cells remain *in situ* and continue as embryonal peritoneal epithelium. The latter early (in 1.5- to 2.0-mm embryos) becomes reduced to a simple single-layered thick epithelium whose cells may range from squamous to cuboidal in shape At the same time its heteroplastic capacity to produce migratory cells becomes progressively restricted to select areas At certain

[illegible]

of such peritoneal epithelial extensions eventually become transformed directly into the physiological parenchymal cells of the spleen, the gonads, the adrenal cortex, the oviducts, and the uterus (Arey, 1954, Jordan, 1925, Richter, 1953, Patten, 1947).

In a human embryo of the seventh week the primitive spleen consists of a close-meshed epithelioid network of cells in anatomical continuity with the peritoneal epithelium (see FIGURES 8 and 12, Thiel and Downey, 1921, Holyoke, 1936) and is the primitive reticulum cell population or initial splenic parenchyma (Thiel and Downey, 1921, Holyoke, 1936) According to Thiel and Downey (1921) and the majority of others it becomes transformed directly into the fixed reticulum cell component of the definitive spleen It should be emphasized that the splenic reticulum cell component is the *only* reticulum cell population in the body that can be traced in unbroken anatomical continuity from the earliest populations of mesoblast cells

On the basis of this brief resumé of the cytology of the early extraembryonic and embryonic human mesoblast and, in particular, of the developmental relation of the latter to the peritoneal versus the mesenchymal cell lines, the following points bearing specifically on the RES problem seem warranted

(1) It is of fundamental significance, rather than mere fortuity, that the physiological parenchyma of the spleen, gonads, adrenal cortex, and oviduct-uterus complex should all have similar developmental origins from the embryonal peritoneal epithelium and, through the medium of the latter, be traceable in unbroken histoanatomical continuity from the earliest populations of human embryonic mesoblast cells

(2) The gonads, the adrenal cortex, the spleen, and the oviduct-uterus complex, as a collective group (a) are functionally interdependent and (b) have effects on the diffuse mesenchymal cell line derivatives that are consistent with and reflect their common developmental origins from primitive embryonic peritoneal epithelium Splenic function, for example, influences the cytogenic activity of the hemopoietic tissues of mesenchymal cell-line origin that have unquestioned embryonic qualities (Doan, 1957, and others), and it also influences the endometrial decidual reactions, a phenomenon involving cells of

mesoblast, including the cells and intercellular substance, is delimited as a unit from the adjacent ectodermal and entodermal layers by a limiting or basement membrane of sorts (Krafka, 1941) except in the area of the primitive streak with which the mesoblast layer is continuous.

The embryonic mesoblast of a 17- to 25-day ovum. As is well known, the loosely textured embryonic mesoblast layer shown in the 14-day ovum (FIGURE 3) becomes highly cellular and epithelioid in character (Torrey, 1954) and, in the region of the prospective trunk, it becomes divided into a somite and a lateral mesoblastic sheet (FIGURE 5) The latter, through the development of an embryonic coelom within it is converted into 2 mutually continuous layers of peritoneal epithelium, one of which is adjacent to entodermal epithelium and the other adjacent to embryonic ectodermal epithelium (see Torrey, 1954, and FIGURE 5) It is comprised mainly of tall, slender, somewhat spindle-shaped, bipolar cells whose proximal processes abut on the peritoneal coelom and whose distal-branched processes collectively form a lose meshwork extending to the adjacent entodermal and ectodermal epithelia (FIGURE 6). Their cytoplasm is extremely basophilic, and their nuclei are equally hyperchromatic (FIGURE 6) This cell type is mitotically active, and especially so are those situated in the region of continuity of the parietal and visceral peritoneal layers (FIGURE 6) They are highly pluripotential cells, and are clearly the base stock for at least two (a third could be the nephric cell line in man—see Torrey, 1954) great genealogical lines of cells that are differentiated and produced within the early, primitive peritoneal epithelium, namely, (1) the mesenchymal cell line, and (2) the peritoneal cell line Both lines in this ovum can be simply distinguished on an histological or cellular behavioral basis The mesenchymal cell line is comprised of a group of migratory cells that give up their positions within the primary peritoneal epithelium (FIGURE 6) The peritoneal cell line is comprised of that group of cells of the primary peritoneal epithelium that do not migrate, but remain *in situ* and persist

to round form, with homogeneous or vacuolated cytoplasm with round to oval nuclei with one or two prominent nucleoli (FIGURE 6)

In the course of embryonic events the cells of the migratory or mesenchymal cell group collectively become transformed into the loose mesenchymal connective tissue cells, vascular endothelium, blood cells, and other such entities (FIGURE 9). They, also, are the cells whose precise subsequent developmental fate is difficult to follow and which have occasioned so much controversy in the literature Their precise developmental fates are obscure because their

pointed out that as the

(FIGURES 5 and 6) are morphologically dissimilar to the later conventionally

the maintenance of normal functional and morphologic organ integrity, whether the object be a lymph node, ovary, urinary bladder, or other structure. Their parameters, therefore, during cultivation by any means become of major concern and merit closest scrutiny when attempting to assess the role of the several morphologic components in the pathogenesis of particular clinical lesions (Altschuler and Angevine, 1957), a role that we are attempting to assess from many approaches in this monograph on the Hodgkin's lesion.

The over-all objective, therefore, in this section is to refocus our perspective on the parameters of some of the inflammatory tissue responses obtainable *in vitro* that in the past have variously influenced our views on several facets of the RES problem and, second, to do this somewhat comparatively by a consideration of the responses of specific tissues as expressed in organ-fragment cultures and intact-organ cultures

Relative to the technical methods employed, only a few points need be [illegible]

and slightly modified organ culture techniques of Chen (1954), Fell (1953), and Trowell (1953) were used. Third, the fluid nutrient phase employed consisted of mixtures of human ascitic fluid, 8- to 10-day chick embryo extract and a physiological saline (Earle's, Simm's, Tyrode's) Finally, the physical substrate was provided by a chicken plasma clot for organ fragment explants and by siliconed lens paper floats for organ explants

Explanted tissues from which data has been drawn include, on the one hand, intact urinary bladder, adrenal gland, and ovaries of the adult bat *Tadarida mexicana* and intact lymph nodes of the 3-month-old (sexually mature) white mouse (Carworth Farms Strain No 1) and, on the other hand, organ fragments of adult human lymph node, perirenal fat body, urinary bladder, anterior pituitary gland, and embryonic chick heart.

Prefatory to a consideration of specific tissue responses of organs and organ fragments in culture, several general aspects should be stressed

(1) The tissue responses in both types of cultures are the consequence of tissue insult (McCutcheon, 1953) and their release is most effectively accomplished by cultivation of organ fragments wherein the degree of insult is great because of the technical necessity of cutting the donor organ into suitably small pieces, that is, 1 0 mm thick and less (Fell, 1953) The intact organs providing data for this report are naturally small (up to 1 0 to 1 5 mm thick) and require no cutting, so the degree of tissue insult is materially lessened

(2) There are marked quantitative (demonstrable, but not precisely measurable), qualitative, and organologic differences between the tissue responses of organ fragments and intact organs in culture

(3) In cultures of organ fragments, the organotypic structure (Maximow, 1925) usually becomes obscured and, even though isolated histotypic differentiations (Maximow 1925) may occur, they do so in uncontrolled manners In cultures of organs, as done along the methods indicated, the organotypic relations of the several constituent tissues and structures are for the most part

generally accepted embryonic character and of close relation to the peritoneal cell line (see Rigler, 1956, and FIGURES 1, 2, 10, and 11). The adrenal cortex and gonads (through the medium of the steroids produced) have specific influences on the general lymphoid tissue and ordinary connective tissue elements of the mesenchymal cell line (Anderson *et al.*, 1951; Aldrich *et al*, 1952; Barber and Delaunay, 1951; Bloom, 1952, Gillette and Buchsbaum, 1953, Grossfeld and Ragan, 1954; Heilman, 1945; Holden *et al.*, 1953, Kaufman *et al*, 1953; Leahey and Morgan, 1952; Mancini and Sacerdote de Lustig, 1951; Miller, 1954, Montgomery and Green, 1954; Paff and Stewart, 1953; Pomerat *et al*, 1949, Ragan, 1954; Ruskin *et al*, 1951, Schneider and Horstman, 1952; Steen, 1951; Trowell, 1953; Speirs, 1955; Gordon, 1937; Marine *et al*, 1924, and others)

(3) Peritoneal epithelium is a separate structure, embryonally and definitively (Stout and Murray, 1942, Chlopin, 1931, Iversen, 1957), and under no circumstance should it be considered as a layer of flattened fibroblasts or even mesenchymal cells of the conventional type (FIGURE 9) As so many investigators have done in the past (Lewis, 1923a, 1923b; Schott and Weidenreich, 1909, Cunningham, 1922a, Clarke, 1915–1916; Holyoke, 1936).

(4) The fact that the reticulum cell population of the spleen, which is the single largest aggregate of this tissue type in the body (Doan, 1957), should very early in the course of embryonic development be set apart from that which develops much later and quite obscurely from the ordinary diffuse mesenchymal cell line, confirms the likelihood of functional possibilities for the spleen (Gordon, 1937, Rigler, 1956) beyond those related to phagocytic sequestration of blood cells and hemopoiesis (Doan, 1957) The straight-line developmental origin of splenic reticulum cells from the earliest pluripotential embryonic mesoblast cells and the cytomorphic likeness of prevascular extraembryonic mesoblast cells and reticulum cells of adult lymphoid organs affirm the generally accepted view that the adult reticulum cell is essentially embryonal in capacity and character

The Morphologic and Functional Responses of Some RES-Implicated Cell Types and Tissues in Vitro

The importance of the RE system in inflammation *in vivo* has been well recognized for many years (Rebuck and Crowley, 1955, Aegerter and Long, 1949, Altschuler and Angevine, 1957) It has stimulated the initiation of many *in vitro* studies (Murray and Kopech, 1953) via the implementation of (1) [illegible]

[illegible] successful *in vitro* culturing must be dependent upon the release of normal intrinsic inflammatory processes within the explant (McCutcheon, 1953, and others) Such processes *in vitro* comprise the basic mechanisms effecting

lium—epithelia of early diverging genealogical and developmental lines (see section on mesoblasts, above)—are similar (compare FIGURES 19 and 21). They are easily confused in organ-fragment cultures wherein organotypic relations are lost or not sought (Congdon, 1915, Lewis, 1923a, 1923b).

The role of coelomic epithelia in the formation of phagocytic exudate cells and the question of whether they have a phagocytic capacity have been discussed for many years (Cunningham, 1922a, 1922b, 1922c, 1924, Maximow, 1927; Foot, 1921; Sampaio, 1956). There seems to be no general disagreement that serosal epithelial cells can segregate colloidal particles both *in vitro* and *in vivo*. My studies of organ-cultured adult urinary bladders indicate that serosal cells may phagocytize cellular debris also (FIGURE 14), whether they become free histiocytes is unsettled. In bladder organ-cultures, the serosal epithelial cells may become round and project above the peritoneal surface from slender anchoring cytoplasmic stalks. Cunningham (1924) has reported similar behavior *in vivo*. Whether this is a phase of transformation into free exudate cells has not been determined, although, when one considers the small amount of serous fluid present in the body cavities under normal circumstances, the stalked arrangement of the serosal epithelial cells could be of distinct functional advantage in phagocytosis, without the necessity of their becoming free cells. In view of the several different tissue sources which, it has been claimed, give rise to the phagocytic exudate cells in inflammation (Cunningham, 1924), it should be stated that there is no evidence thus far from any of my serosal-surfaced organs in organ culture of excess production anywhere within them of fixed tissue histiocytes or free macrophages or of an exodus of macrophages. It is interesting to note that the claim made by Foot (1921), that mesothelial cells are phagocytic and probably perform the same function for the serous cavities as do the endothelial cells, is rather exemplified in organ-cultured bladders where both mesothelial and endothelial cells have been found to contain cellular debris (FIGURES 14 and 17).

In summary, it seems necessary to say that the coelomic epithelia *in vitro* (1) have the capacity for normal histotypic and organotypic self-maintenance, (2) that their cells have a high pleomorphic capacity, (3) that they have the capacity, even though normally latent or restricted, to phagocytize cellular debris and colloidal particles, and (4) that their role, if any, in the production of phagocytic serous exudate cells remains to be determined.

In vitro response of the adrenal gland. In view of the known developmental (see section on human mesoblasts, above) and functional relations between the adrenal cortex, spleen, and other lymphoid organs (White and Dougherty, 1946, and many others) and, second, because of the unsettled state of knowledge regarding the mechanism of regeneration of adrenal cortical epithelium (Schaberg, 1955, Gonzalez, 1955), it seems desirable to comment briefly on certain of our findings (Richter *et al.*, 1957) on the response of the adult adrenal gland of the bat in organ culture.

During cultivation the adult gland acquires a relatively thick capsule of fibroblastlike cells (Schaberg, 1955). They are mitotically active and have a close similarity to the general peritoneal epithelial cells, even during mitosis

retained, and specific tissue responses are usually very orderly, organized, and controlled, so that difficulties of tissue and cell identification are lessened materially. For instance, after many days of cultivation the end product may be an ovary or lymph node that looks like an ovary or lymph node and apparently functions in part as such (FIGURES 50 and 51)

(4) Basic cellular capacities, centering about such protoplasmic phenomena as motility, pleomorphism, phagocytosis, secretion, hypertrophy, growth, and mitosis, which *in vivo* are held in abeyance, somehow concealed or normally compensated for by the activities of other cell types or intercellular substances, are frequently released by these methods of culturing. Consequently, various degrees of exaggerated states of hypertrophy, phagocytosis, pleomorphism, and so on may be attained by cells *in vitro* that normally are attained *in vivo* never or only seldom. The degree of exaggeration is generally greatest in cultures of organ fragments and minimal in cultures of intact organs.

(5) Of signal importance is the point that in the intact organ culture methods used, tissue responses are not obscured by the mobilization of blood-borne leukocytes

In vitro responses of coelomic epithelial cells. The coelomic epithelium generally, regardless of its specific designation as germinal epithelium, or general mesothelium of pleural, pericardial, or peritoneal cavities, is capable of maintaining itself homoplastically both *in vitro* and *in vivo* (Cunningham, 1924) *In vivo* this capacity is manifest occasionally in mesotheliomatous growths (Stout and Murray, 1942) and in experimentally induced serous inflammatory processes (Cunningham, 1924)

In vitro, its growth response varies quantitatively and morphologically. In cultures of intact adult ovaries, oviducts, and urinary bladders (Richter *et al.*, 1956), the surfacing germinal and general peritoneal epithelia are repaired homoplastically through mitosis and retain their normal histotypic structure and organotypic relations (FIGURES 13 and 16). There is quantitatively no

[illegible]

1956, and others)

In contrast, homoplastic growth of coelomic epithelium in organ-fragment cultures may result in massive outgrowths having modified histo-organotypic patterns (Gaillard, 1950), or histotypic sheets (FIGURE 19) or variously disorganized sheets (Rosin, 1947, Chlopin, 1931) whose constituent cells show diverse kinds and degrees of hypertrophy and pleomorphism (see FIGURE 19, Ivers and Pomerat, 1947, Stout and Murray, 1942) Such histotypic and cellular pleomorphic deviations undoubtedly have been the basis for conflicting reports that mesothelial cells and/or fibroblasts mutually transform from one into the other (Lewis, 1923a, 1923b) and moved Bloom (1937) to comment on the handicaps imposed on cellular identification by pleomorphic changes Well-organized histotypic growths of cardiac mesothelium and cardiac endothe-

lium—epithelia of early diverging genealogical and developmental lines (see section on mesoblasts, above)—are similar (compare FIGURES 19 and 21). They are easily confused in organ-fragment cultures wherein organotypic relations are lost or not sought (Congdon, 1915, Lewis, 1923a, 1923b).

The role of coelomic epithelia in the formation of phagocytic exudate cells and the question of whether they have a phagocytic capacity have been discussed for many years (Cunningham, 1922a, 1922b, 1922c, 1924, Maximow, 1927, Foot, 1921; Sampaio, 1956). There seems to be no general disagreement that serosal epithelial cells can segregate colloidal particles both *in vitro* and *in vivo*. My studies of organ-cultured adult urinary bladders indicate that serosal cells may phagocytize cellular debris also (FIGURE 14), whether they become free histiocytes is unsettled. In bladder organ-cultures, the serosal epithelial cells may become round and project above the peritoneal surface from slender anchoring cytoplasmic stalks. Cunningham (1924) has reported similar behavior *in vivo*. Whether this is a phase of transformation into free exudate cells has not been determined; although, when one considers the small amount of serous fluid present in the body cavities under normal circumstances, the stalked arrangement of the serosal epithelial cells could be of distinct functional advantage in phagocytosis, without the necessity of their becoming free cells. In view of the several different tissue sources which, it has been claimed, give rise to the phagocytic exudate cells in inflammation (Cunningham, 1924), it should be stated that there is no evidence thus far from any of my serosal-surfaced organs in organ culture of excess production anywhere within them of fixed tissue histiocytes or free macrophages or of an exodus of macrophages. It is interesting to note that the claim made by Foot (1921), that mesothelial cells are phagocytic and probably perform the same function for the serous cavities as do the endothelial cells, is rather exemplified in organ-cultured bladders where both mesothelial and endothelial cells have been found to contain cellular debris (FIGURES 14 and 17).

In summary, it seems necessary to say that the coelomic epithelia *in vitro* (1) have the capacity for normal histotypic and organotypic self-maintenance, (2) that their cells have a high pleomorphic capacity, (3) that they have the capacity, even though normally latent or restricted, to phagocytize cellular debris and colloidal particles, and (4) that their role, if any, in the production of phagocytic serous exudate cells remains to be determined.

In vitro response of the adrenal gland. In view of the known developmental (see section on human mesoblasts, above) and functional relations between the adrenal cortex, spleen, and other lymphoid organs (White and Dougherty, 1946, and many others) and, second, because of the unsettled state of knowledge regarding the mechanism of regeneration of adrenal cortical epithelium (Schaberg, 1955, Gonzalez, 1955), it seems desirable to comment briefly on certain of our findings (Richter *et al.*, 1957) on the response of the adult adrenal gland of the bat in organ culture.

During cultivation the adult gland acquires a relatively thick capsule of fibroblastlike cells (Schaberg, 1955). They are mitotically active and have a close similarity to the general peritoneal epithelial cells, even during mitosis

(FIGURES 13 and 15). After 19 to 21 days in culture, small organized epithelioid nodules and centrally directed cordlike structures are formed within the capsule (FIGURE 52); they appear to be differentiating cortical epithelial cell structures These findings are consistent with those of Schaberg (1955) on the mouse adrenal in culture and would tend to support the view relating some cortical cell regeneration to the capsule

In vitro response of common vascular endothelium. The phagocytic, proliferative, and developmental potentiality of the common vascular endothelial cell is yet unsettled, and is today the subject of almost diametrically opposed views (Altschul, 1954, Policard, 1957, Doan, 1957, Jaffe, 1938; Toro, 1947).

In a wide assortment of intact organs from the adult bat and mouse in organ culture, the major histo-organotypic alteration from normal that has been observed is the disappearance of conventional blood cells of all types (except extravascular connective tissue eosinophils) and of capillaries

Blood cells, present in the vessels at the time of organ explantation, within a few days undergo such processes as nuclear confluence, pyknosis, and autolysis, and die within the vessels, as observed *in vivo* in vessel ligation (Richter, 1942, Clark and Clark, 1936) Many of them, however, may be phagocytized by the local common vascular endothelium The cells of the latter, although slightly hypertrophic and containing rather considerable amounts of cellular debris, do not give up their positions within the endothelium (FIGURE 17). An extravascular migration of the stranded blood cells has not been observed. A similar removal of peripheral blood elements occurs in the special venous [illegible] cytologic, histologic, nor organologic evidence that the phagocytic endothelial cells present are anything other than common vascular endothelial cells in which a latent capacity to phagocytize has been released under the mild inflammatory stimulus of the hemostasis occasioned by the method of organ cultivation (Jaffe, 1938). Perhaps the differences between ordinary and special endothelial cells, such as the Kupfer cells and those of certain other venous sinusoids, are not as distinct as we are inclined to think and are more of the nature of different degrees of expression of functional capacity than of fundamental generic difference (Jaffe, 1938) It seems evident that adult, fully differentiated common vascular endothelium has the basic capacity to take up both colloidal particles (Jaffe, 1938, Maximow, 1928) and also fragments of worn-out blood cells under appropriate aseptic inflammatory circumstances The latter cannot be dismissed as an endothelial "transfer process," as has been done relative to colloidal particles (Jaffe, 1938), it is a real phagocytic process and, morphologically, precisely like that observed in the most "bona fide" fixed macrophages in other situations (FIGURE 57)

Whether endothelial cells can migrate independently into extraendothelial connective tissue positions, which means an abandonment of their parenchymal vascular status (Altschul, 1954), is the crux of the disagreement regarding the

role of vascular endothelium in inflammation (Doan, 1957; Toro, 1942, 1947; Jaffe, 1938, Cunningham, 1924; Altschul, 1954, Mollendorff, 1933, Maximow, 1928, Policard, 1937). The answer to this question hinges on other specific capacities of the common vascular endothelial cell, such as its pleomorphic and motility capacity and the capacity of endothelium for histotypic and organotypic homoplastic self-maintenance. In organ cultures, it seems evident that vascular endothelium has a high capacity to maintain its normal histotypic and organotypic character, and that the latter is, in part at least, effected by quantitatively controlled and orderly mitoses (FIGURE 18) Indicative of the orderliness by which normal organotypic character is maintained is the precise orientation of the dividing cell, as indicated by the cleavage plane, which is of such nature that the resulting daughter cells occupy positions within the simple endothelial lining (FIGURE 18) This orderly histotypic and organotypic self-maintenance of endothelium frequently is observed in capillaries situated deeply within organ-fragment cultures even after long periods in cultivation (FIGURE 23) However, maintenance of the simple squamous type endothelium may be lacking near the traumatized severed ends of larger vessels cut during organ explantation In these areas the endothelium thickens, the constituent cells partially separate, acquire descriptively fibroblastlike appearances, and migrate littorally in the lumen of the vessel and out through any break in the vascular wall or through the open end of the vessel They are not fibroblasts, but merely pleomorphic migratory endothelial cells responding to excessive local tissue trauma and physical tissue disorganization This response in every way is like the quantitatively uncontrolled histotypic growth of endothelial sheets (FIGURE 21), and streamers of fibroblastlike endothelial cells growing from the cut ends of capillaries in cultures of organ fragments from diverse sources (FIGURES 20 and 22—see also Maximow, 1928, Lewis, 1922, Fulton *et al*, 1949)

In view of the demonstrable organotypic growth capacity of vascular endothelium generally, it would seem that the general disappearance of capillaries in intact-organ cultures, as mentioned earlier, is more apparent than real It seems that the capillaries simply collapse through the loss of plasma, and that the capillary endothelium thus acquires the characteristics of slender strands of attenuated cells that are descriptively like those comprising the growing tips of capillaries commonly seen in organ-fragment cultures (FIGURE 22) In these latter instances, were it not for their demonstrable histologic continuity with organized capillaries, the leading endothelial cell strands (FIGURE 22) would be virtually impossible to identify or to distinguish from strands of fibroblasts Maximow has reported (1924, 1928), relative to these endothelial strands in organ-fragment cultures, that the cells gradually move away from each other, develop side processes and, after a while, cannot be distinguished from typical fibroblasts, and that the only new cell type the endothelial elements can produce is the fibroblast This difficulty is also of primary importance in distinguishing between primitive angioblasts (Hertig, 1935) and primitive connective tissue cells in those very early stages preceding blood vessel formation in the extraembryonic mesoblast of human embryos

(FIGURES 1 and 7). I agree that a point may be reached in both organ and organ-fragment cultures, as well as in the earliest embryonic stages, wherein mixed cell types are present in rather disordered array, that endothelial (and angioblastic) cells are quite indistinguishable from fibroblasts, however, at the same time our inability to disting[illegible]

The bulk of early embryonic evidence (Hertig, 1935) indicating separate vascular and extravascular cell lines and the remarkable pleomorphic and motility capacities of endothelial cells, plus their evident capacity for histotypic and organotypic self-maintenance in both organ and organ-fragment cultures, attest rather to the probability that endothelial cells represent a specific fully differentiated generic cell type having a latent or somehow restricted capacity to phagocytize.

Evidence as to whether the common endothelial cells, which in these studies have been shown to possess a true phagocytic capacity, are able to acquire the morphologic characteristics of the conventional histiocyte directly or indirectly, as many have reported (Toro, 1942, 1947, Cunningham *et al*, 1925; Doan, 1957, Jaffe, 1938, Doljanski, 1930) has not yet been found in any of my *in vitro* material However, collectively considered, all data indicate that common vascular endothelial cells are potentially phagocytic migratory cells in their own right, irrespective of the question as to whether they can transform into monocytes and histiocytes.

In vitro response of lymph nodes The responses of lymph nodes *in vitro* [illegible] neral [illegible] rgan culture, the mice used were given daily intraperitoneal and subcutaneous injections of trypan blue for several days This was followed by a stabilizing period of several days without injections, after which the animals were sacrificed for removal of the nodes

Such intact nodes in organ culture may maintain their organotypic and histotypic characters for undetermined long periods of time (Trowell, 1952) There is no general disorganized or quantitatively uncontrolled growth of any of the several specific tissue types present, either within or associated adventitially with the nodes (FIGURES 50 and 51) Histotypic and organotypic characters are maintained, at least in part, by obvious homoplastic growth, as revealed by the presence of limited numbers of mitosing cells in nearly all specific tissue types and tissue structures, including the fixed lining cells of the lymph sinuses (FIGURE 58) as well as the extrasinusoidal fixed reticulum cells (FIGURE 59)

Consistent with the findings in other organs in culture and with the same type of media and other culturing conditions, the lymph nodes also show a progressive degeneration and disappearance of peripheral blood cells However, in the nodes, this phenomenon also involves the free cells in the extrasinusoidal lymphoid cords and nodules and in the lymph sinuses as well

Diminution of the lymphoid free cell population is not due to a mass exodus of cells, as only occasional free lymphoid cells are found out of their normal histologic positions (FIGURES 50, 51, 56–59; Trowell, 1952) Many of them simply undergo pyknosis, lysis, and so on *in situ* (FIGURES 56–59) Many are removed through phagocytosis by the lining sinusoidal cells (FIGURES 57–59, Trowell, 1957).
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the colloidal dye (FIGURES 54 and 55) The phagocytic and the generally retrograde processes involving the free cell population frequently resulted in the conversion of lymphoid cords and nodules into rather close-meshed net-

character (FIGURES 58 and 59)

The actively phagocytic and dye-storing fixed cells of the lymph sinuses were generally slightly hypertrophic and somewhat rounded, and were descriptively of two general types. those having a single large polymorphic nucleus, and those with several normal-sized nuclei Similar hypertrophic sinusoidal cells were not found in the control uncultured nodes (FIGURES 53–57).

The responses of human lymph node fragments in culture are in marked contrast in nearly every way to those of the intact organ-cultured mouse nodes The growth responses of the principal constituent tissue types are quantitatively uncontrolled, great, and disorganized (through loss of organotypic arrange-

1944, 1949, Grand and Cameron, 1946, Lewis, 1941, Lewis and Webster, 1921a, 1921b, Maximow, 1922, Meier *et al*, 1937, Rottino, 1949b)

The ordinary stromal connective tissue component during cultivation of lymph node fragments produces heavy obscuring outgrowths of fibroblasts that may completely dominate the culture and give rise to extensive but unorganized meshworks of fibers (FIGURE 42) The fibroblasts are extremely hypertrophic relative to their prototypes *in vivo*, that is, to fibroblasts in uncultured portions of donor nodes (FIGURE 44) and also to fibroblasts of the organ-cultured mouse nodes This hypertrophic tendency is characteristic of fibroblasts growing from any organ fragment in culture, whether it be a fragment of anterior pituitary gland (FIGURE 43) or connective tissue from the crest of the ilium (FIGURE 45) It is typical, also, of the fibroblasts *in vivo* in inflammation (Altschuler and Angevine, 1957) but, even so, it is less than *in vitro*

The response of the fixed reticulum cells (FIGURE 32) of the parenchymal lymphoid [illegible]
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constituent cells are equally similar. However, unlike the fibroblasts, the reticulum cells, when comprising histotypic formations like that illustrated, are not hypertrophic relative to their prototypes *in vivo*, nor are they characteristically associated with lymphocytes or other lymphocytoid cells as *in vivo*. In the course of cultivation some reticulum cells retract their processes in various degrees and transform into rounded cells which, through the medium of repeated nuclear divisions, hypertrophy into giant multinucleate rounded cells (FIGURES 34–37, 40, 41). Others transform into spindle-shaped or slightly stellate cells that hypertrophy without nuclear division into giant mononuclear stellate cells (FIGURES 34–39). Neither the giant nor the larger transitional forms of these two types were found *in vivo*. All of these variously hypertrophic cells have been observed to phagocytize bacteria accidentally introduced into cultures (FIGURES 37, 40, 41).

The identities and implied genetic interrelations of these two descriptively different hypertrophic cell forms (FIGURES 34 to 41) have been variously reported in the literature on cultured nodes. These cell forms have been identified as fibroblasts, phagocytic fibroblasts, clasmatocytes, fusion products of mononuclears, Reed-Sternberg cells, transitional forms between reticulum cells and large lymphocytes and monocytes, hypertrophic reticulum cells, transformed endothelial cells, and giant cells of Langhans type (Ackerman *et al.*, 1952, Maximow, 1922, Lewis and Webster, 1921a, Lewis, 1925, 1927; Meier *et al.*, 1937, Rottino, 1949b, Trowell, 1955, 1957, Grand, 1949). It seems significant, not only to the problem of their identity and genetic relation, but to [illegible]

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occur in exaggerated forms in organ-fragment cultures of normal nodes and nodes with a wide assortment of lesions other than the Hodgkin's lesion (FIGURES 35–41, Ackerman *et al.*, 1952, Maximow, 1922, Lewis and Webster, 1921a, Meier *et al.*, 1937).

Analysis of these several papers indicates that, quite surely, these workers were seeing members of the same groups of hypertrophic cells as illustrated here (FIGURES 34 to 41) and that, in nearly every instance, their published illustrations showed lymphocytes to be characteristically associated with them as illustrated here. It has not, however, been generally appreciated relative to the second point that this association is quite consistently an association [illegible]

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depressions of the surfaces of the hypertrophying cells (FIGURE 38) as first described by Lewis (1925). Such pockets are not digestive vacuoles, as they do not concentrate neutral red. The lymphocytes are not within the cyto-

plasm of the hypertrophic cells, they are merely enclosed by it (Lewis, 1925) As shown in this illustration, many lymphocytes situated in such pockets eventually die and undergo lysis *in situ* Some actually may be phagocytized, but it is difficult to be sure of this The fate of the lytic products is not known,

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whether the direction of action always progresses from the lymphocyte toward the hypertrophying cell or from the latter cell toward the lymphocyte, or both. This facet of the problem deserves much critical study for several reasons, among which are the circumstances that Reed-Sternberg cells are essentially hypertrophic cells, and that advanced Hodgkin's lesions are characterized by a diminution of the lymphocytic free cell population (M. N. Richter, 1953). This association may have another significance, it may indicate that the several descriptively different hypertrophying cells (FIGURES 34 to 41) have a certain genetic sameness and may be genetically a single cell species, namely, hypertrophic reticulum cells, as I descriptively have treated them here. The mechanism behind this association also may be involved in the general localization of lymphocytes within the lymphoid organs and in other sites as well

Relative to the lymphocytic population of lymph node fragments in culture, lymphocytes of several varieties have been observed in mitosis for periods of up to two weeks, and living lymphocytes have been observed in culture through forty days (FIGURES 37, 40, 41) The majority show progressive retrograde changes resulting in cell death and lysis No positive compelling evidence has been found to indicate that the lymphocytes produce any cells other than lymphocytes (Ehrich, 1946)

In vitro responses of human mast cells The mast cell, although deeply implicated in the inflammatory process (Asboe-Hansen, 1957, Ragan, 1954;

ture is almost completely limited to that of Zitcer *et al* on material obtained from an infant with urticaria pigmentosa The observations to be reported are based on explants from the urinary bladder of an adult male whose case was diagnosed as a chronic mucinous inflammatory reaction to squamous metaplasia

My observations on the mast cells of adult tissue in culture are in accord with those of Zitcer *et al* on the infant skin, although the extensive outgrowths of mast cells obtained by them were lacking in my cultures, due in part, perhaps, to initial differences in mast cell populations characterizing these two lesions Explants after 29 days in culture showed no outgrowths of any cellular component except mast cells (FIGURE 61) Their cytomorphology ranged

from essentially rounded cells of varied sizes densely filled with metachromatic granules (FIGURE 60) to rather hypertrophic, pleomorphic cells sometimes of fibroblastic shape and sometimes with long, slender dendritic-type processes exceeding 80 μ (FIGURE 61) In extended, motile forms, the granules were dispersed throughout the cell body and processes and were of diverse sizes. Only occasional vacuoles were noted. Time-lapse motion picture records showed that they exhibited a fibroblastlike movement and were capable of dividing amitotically. It has been reported that size increase of the mast cell may represent a developmental sequence (Sundberg, 1955). Relative to this point, it was noted in the main body of the explants that the smallest mast cells were intimately associated perivascularly with postcapillary venules and the larger ones with the larger arterioles and venules (FIGURE 60). Whether this indicates some heteroplastic formation of mast cells from primitive perivascular tissue could not be determined, although it is suggestive of it (Ragan, 1954, Maximow, 1927; Sundberg, 1955).

While the relation of mast cells to the mucopolysaccharides of connective tissue ground substance is not surely known, it is interesting in view of Asboe-Hansen's work (cited in Ragan, 1954) to note that, even though mast cells are reported to be present normally in large numbers in the urinary bladder (Sundberg, 1955), mast cell growth has been obtained only in cultures from a bladder showing a mucinous inflammatory reaction. Many hundreds of cultures have been studied from urinary bladders with other lesions, mainly neoplastic (Richter and Akin, 1957)

It would seem that the responses of mast cells in organ-fragment cultures are consistent with those found in connection with others relative to uncontrolled growth and exaggerated cellular pleomorphic and hypertrophic tendencies

In vitro response of fat cells and fat tissue It is generally agreed that the fat cell, whether of brown or white variety, is of mesenchymal origin (Sidman, 1956, Murray and Stout, 1943, Stout, 1944, Burkhardt, 1934, Maximow, 1927, Chevremont, 1948) What its relation is to other cell types in fully differentiated fat tissue is not clear Chevremont (1948) visualizes the fat cell as a fat histiocyte arising from a mesenchymal or fibroblastic cell type Stout (1944) regards it as arising from a common ancestral cell type segregated from the primitive mesenchyme and possessing certain metaplastic capacities Wells (1940) views fat cells as being closely related to reticulum cells, lymphoid tissue, and perivascular mesenchymal cells Burkhardt (1934) views them as remarkably pleomorphic and phagocytic cells reflecting certain mesenchymal cell characteristics It is generally agreed from *in vivo* studies (Wells, 1940) that fully differentiated signet-type fat cells do not undergo mitosis, and that immature ones (lipoblasts) with limited or no intracellular fat inclusions do so (Murray and Stout, 1943, Burkhardt, 1934), but it is not clear whether the growth of fat tissue in all situations is the result of increasing the fat cell population by homoplastic or heteroplastic means or both

In organ cultures containing fully differentiated adventitial fat tissue, the latter is extremely stable and retains its normal histologic features for un-

determined long periods (FIGURES 50 and 51). In such fat tissue the signet-type cells have not been observed to mitose, and they remain virtually unaltered during cultivation. Sometimes one or a group of them will show several smaller fat droplets, but in these instances it cannot be determined whether they are accumulating fat or removing it. The stromal connective tissue component remains organized, and limited mitoses occur among its cells. Because of the scarcity of stromal tissue and an abundance of capillaries and blood vessels in organized fat, the mitosing cells are generally perivascularly situated

In marked contrast are the responses of cultured tissue fragments of adult perirenal and other fat tissues Outgrowths are disorganized and are comprised mainly of two types of cells fibroblasts and lipoblasts (Murray and Stout, 1943). The lipoblasts illustrated in FIGURE 24 show a minimum of *differentiation into fat-storing cells in the outgrowth zones. In these loca-*

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cytoplasm (FIGURES 23, 24, and 28) and by nuclear volume differences between the smaller (FIGURE 24) and the larger cells (FIGURES 27 and 28). In many instances the hypertrophic lipoblasts can be traced in streams back into the main body of the explant to small, basophilic wing-shaped and elongate cells situated between fully differentiated stable fat cells, and in perivascular sites (FIGURE 26). In some instances the smaller wing-shaped cells, especially around the blood vessels, show a differential accumulation of fat and acquire the appearance of immature signet-type fat cells (FIGURE 25). They are comparable to the differentiating fat cells occasionally obtained in cultures of embryonic tissue (FIGURES 29 to 31) Mitotic activity has been observed only in the smallest lipoblasts, which demonstrates the homoplastic capacity of the fat cell line The possibility of some heteroplastic growth from another

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perivascular sites (FIGURES 25 and 26) It is interesting to note that Cunningham, *et al* (1925) published a photograph of marrow *in vitro* quite like FIGURE 23 and identified the small mitosing cell as a pericapillary reticulum cell In this connection, the hypertrophic lipoblasts described here, which can be traced rather directly to perivascular and pericapillary sites in the main explant, are frequently found associated with lymphocytes in the same way

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that occasioned Burkhardt (1934) to consider his atypical fat cells as being phagocytic

In vitro response of fibroblasts or fibroblastlike cells to cytotoxic stimuli With

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General Summary

The early cytology and development of the human mesoblast have been presented to establish the specific developmental and genetic basis for the particular functional and morphologic interrelations and attributes characterizing (1) the splenic reticulum cell population, (2) the adrenal cortical parenchyma, (3) the gonadal and oviduct-uterus parenchyma and, possibly, (4) the nephric parenchyma, all of which are deeply involved in the total RES problem.

An attempt has been made to establish, via responses of various RES-implicated organs, tissues, and cells in organ cultures and in organ-fragment cultures, the facts that *in vitro* studies initiate a series of variously exaggerated inflammatory functional and morphologic changes at both the organ, tissue, and cellular levels, and that cognizance of the parameters of these changes at all levels is essential to the establishment of generic relations, of functional interrelations and capacities, and even of morphologic capacities of the several RES-implicated cells and tissues

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the linkage of histamine and heparin to mast cells (Riley, 1953, 1954; Ehrlich *et al.*, 1949), renewed interest has developed in connection with the role of histamine on the morphology and function of connective and other tissues, and has occasioned many *in vivo* and *in vitro* studies involving the use of antihistamine, histamine, and histamine liberators (Chevremont, 1955, Feldberg and Kellaway, 1938; Grossberg and Garcia-Arocha, 1954, Zweifach, in Ragan, 1954; Richter, 1956; Pomerat *et al*, 1949, Riley, 1953, Schachter, 1952, and Toro, 1947).

Histamine in physiological and near-physiological concentrations is known to effect changes in secretory, contractile, and growth processes (Richter, 1956) and to effect capillary integrity and endothelial cell changes (Zweifach, in Ragan, 1954).

It is not generally appreciated that histamine (as well as antihistamine) at toxic concentrations effects a progressive series of rather standard cytopleomorphic transformations eventually resulting in cell death (Richter, 1956, Toro, 1947, Zweifach, in Ragan, 1954), and that these transformations are precisely the same as those elicited by a most diverse group of specific and nonspecific chemical and physical agents, including arsenic, heat, cold, trypan blue (Mollendorff, 1933, Bloom, 1938), atropine, bacteria (Tannenberg, 1930a, 1930b, Muhlethaler, 1952), and poisonous extract from *Houttecynia cordata* (Ueta, 1955) Certain phases of these retrograde cytomorphic alterations are prone to misinterpretation They frequently have been misinterpreted and, insofar as I can determine, they have played key roles in influencing our views on the pluripotential capacity and specific relationship of the common fibroblast to the macrophage or histiocyte (Bloom, 1938, Chevremont, 1955, Toro, 1947, Mollendorff, 1933). Lastly, as histamine is known to institute similar retrograde endothelial cell changes *in vivo* (Zweifach, in Ragan, 1954, Toro, 1947), the validity of at least part of the original work bearing on the endothelial origin of erythrocytes and macrophages (clasmatocytes) may have been jeopardized (Cunningham *et al*, 1925b) as the subject animals were given injections of histamine before sacrifice to effect dilatation of the marrow vessels

During the past five years, studies involving several thousands of cultures have been aimed at an evaluation of the cytophysiologic and cytomorphic responses of cells to histamine, antihistamine, cortisone, adenosine triphosphate (ATP), and glutathione, when administered singly and in all combinations These studies individually and collectively attest to the fact that the various shapes or configurations assumed by fibroblasts (FIGURE 46) which superficially simulate, in sequence, macrophages or histiocytes (compare FIGURES 35 to 37 and 47), lymphoidlike cells (FIGURE 48), and granular leukocytes (FIGURE 49) are retrograde cytomorphic alterations (modulations or adaptations) to toxic or untoward stimuli Such changes and changed fibroblasts are exactly parallel to those associated with the alteration of mature peripheral blood granulocytes into simulated promyelocytic and myeloblastic cells (Richter, 1942) to which the Clarks (1923, 1930) gave the name "dwindle cells" The fibroblasts here could well be called "dwindle fibroblasts"

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PLATES

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PLATES

PLATE I*

FIGURE 1 General view of 11-day human ovum showing trophoblast (tr), extraembryonic mesoblast (xm), basement membrane (bm), entodermal vesicle (ev), amniotic vesicle (av), and embryonic area (ea) H and E † ×339

FIGURE 2 Portion of extraembryonic mesoblast area in FIGURE 1, showing cytologic detail of the 2 principal types of extraembryonic mesoblast cells, small basophilic type and large fibroblastic type H and E ×778

FIGURE 3 Cross-sectional view of lateral half of embryonic area of 14-day human ovum, showing embryonic ectoderm (ee), primitive streak (ps), embryonic mesoblast (em), artifactual separation space (as), embryonic entoderm (ent), and extraembryonic mesoblast (xm) H and E ×339

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× [illegible]

* All illustrations are unretouched photomicrographs taken with the 35 mm Leica camera in combination with the Micro Ibso attachment

† H and E = Hematoxylin and eosin staining

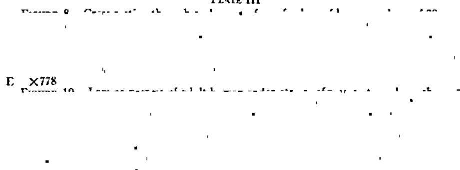

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PLATE II

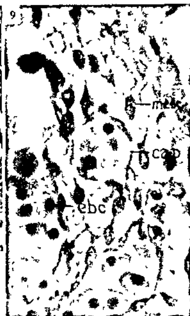
FIGURE 5 General cross sectional view of lateral half of human embryo of fourth week.

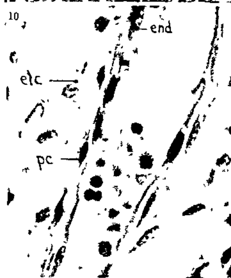

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PLATE III




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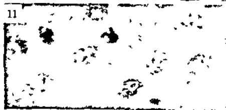

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PLATE IV

FIGURE 13 Twenty-one-day organ-cultured urinary bladder of adult bat, showing organized maintenance growth of peritoneal epithelium with cell in division (mit) H and E ×778

and E ×778

FIGURE 18 Small venule, showing organized maintenance growth of lining endothelium with mitosing endothelial cell (mit) from urinary bladder of adult bat 14 days in organ culture H and E ×778

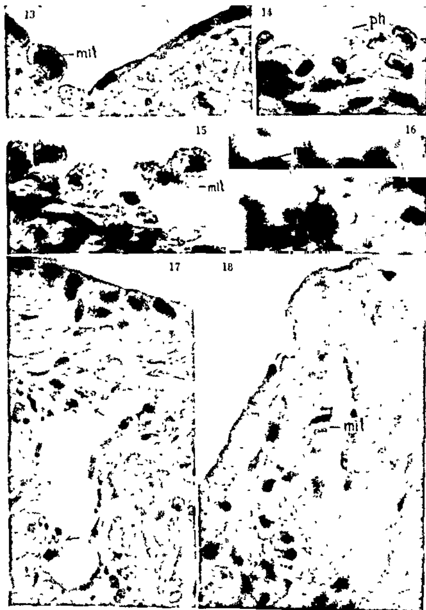
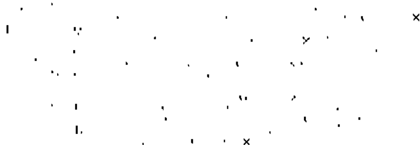

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PLATE V

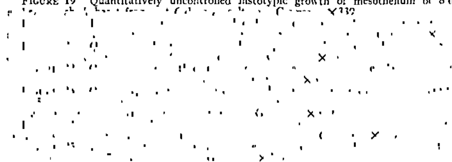
FIGURE 19 Quantitatively uncontrolled histotypic growth of mesothelium of 8-day [illegible]




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fc
mi

PLATE V

FIGURE 19. Quantitatively uncontrolled histotypic growth of mesothelium of 8 day [illegible]



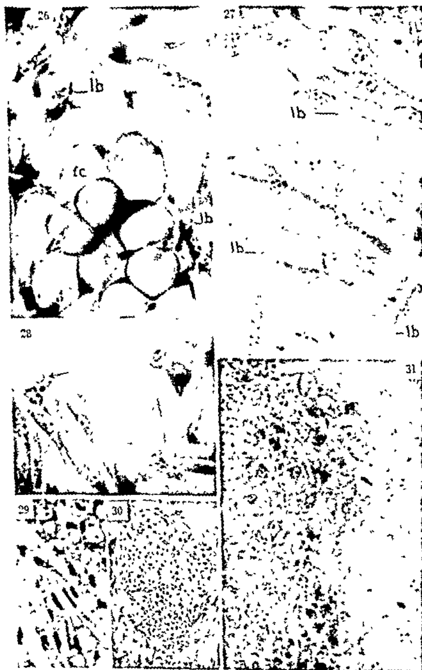

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lb
fc
lb
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lb
lb
lb
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PLATE VI

FIGURE 26 Explant of adult human fat tissue from crest of ilium five days in culture, showing mature fat cells (fc) and small migratory, wing-shaped lipoblasts (lb) Giemsa ×339

FIGURE 27. Disorganized and unrestricted growth of variously pleomorphic and hypertrophying, but nonfat-storing lipoblasts in an explant of adult human fat tissue 5 days in culture Giemsa ×339

FIGURE 28 Variously hypertrophic and pleomorphic lipoblasts with one in lymphocytic association, from adult human fat tissue from crest of ilium, 5 days in culture Giemsa ×339

FIGURE 29 Differentiating embryonal fat cells from 8-day embryonic chick heart 5 days in culture Giemsa ×339

FIGURES 30 and 31 A nodule of homoplastically derived, fat-storing embryonal lipoblasts from 8-day embryonic chick heart 5 days in culture, enclosed by ordinary embryonal connective tissue fibroblasts Giemsa FIGURE 30, ×84, FIGURE 31, ×339

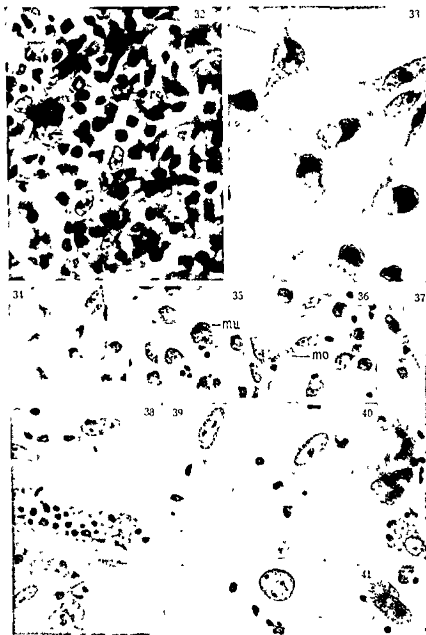
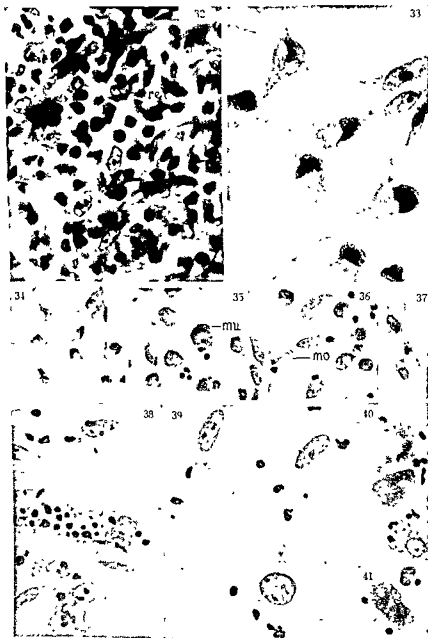

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PLATE VII

FIGURE 37 [illegible]

lymph node (FIGURE 32) Giemsa ×339

FIGURES 40 and 41 Moderate to giant hypertrophic multinucleate forms (like mu of FIGURES 35 and 36) with phagocytized bacteria and in living lymphocyte association from lymph node (FIGURE 32) 40 days in cultivation Giemsa ×339



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PLATE VIII

Giemsa ×

FIGURE 47 Intermediate retrograde changes of 8-day embryonic chick heart fibroblasts 5 days in culture after treatment with ATP and 100 μg of histamine-HCl during days 3 to 5 Note the simulated macrophage appearance of the rounded forms Giemsa ×339


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PLATE IX

FIGURE 51 Uncultured control adult mouse lymph node, compare with FIGURE 50 H and E. ×84

FIGURE 52 Intact adrenal gland of adult bat 19 days in organ culture, showing the differential organization of epithelioid nodules (n) from capsular cells, viable atrophic zone (va), and unviable atrophic (ua) zone of "ghost" cells H and E ×339

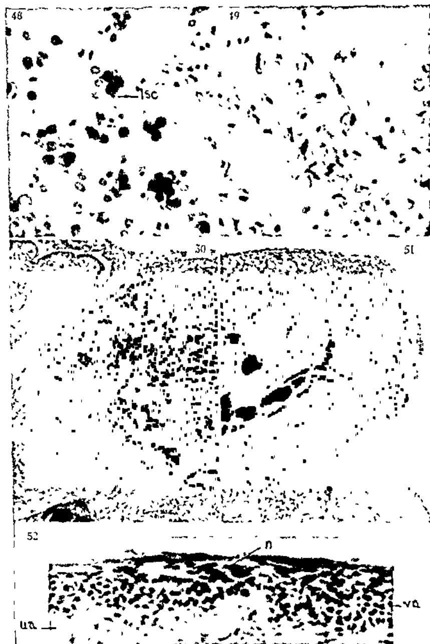

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n
va
ua

PLATE IX

treatment with 100
d glutathione during

FIGURE 50 Intact adult mouse lymph node 4 days in organ culture to show maintenance of normal organ-typic character H and E ×84

FIGURE 51 Uncultured control adult mouse lymph node, compare with FIGURE 50 H and E ×84

FIGURE 52 Intact adrenal gland of adult bat 19 days in organ culture, showing the differential organization of epithelioid nodules (n) from capsular cells, viable atrophic zone (va), and unviable atrophic (ua) zone of "ghost" cells H and E ×339

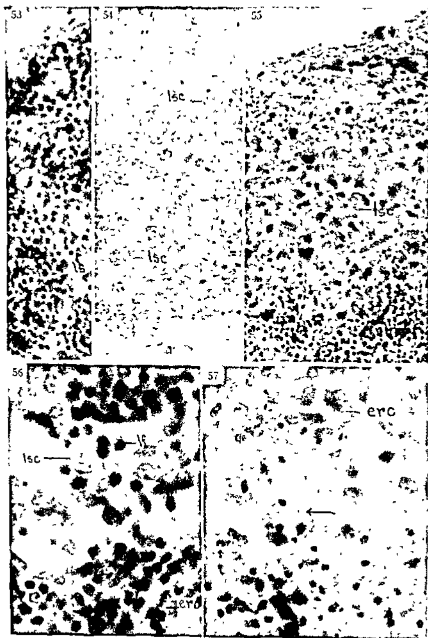

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PLATE X

reticulum cells (erc) H and E ×778

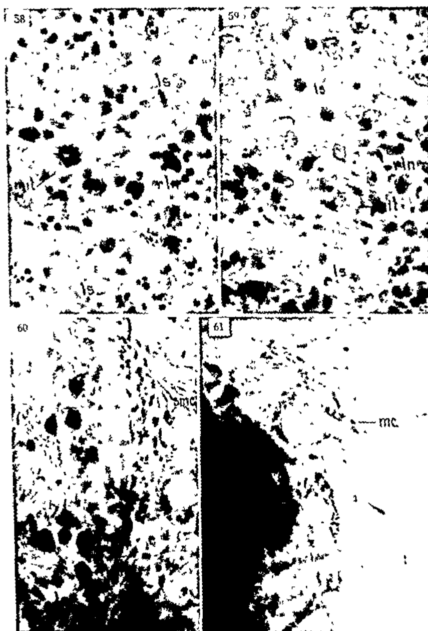
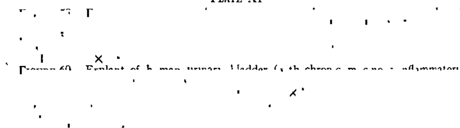

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PLATE XI



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in part, by such factors as the acidic groups, relative concentrations, and degree of polymerization of the mucopolysaccharides, as well as by the complex nature of the other ground substance constituents It is therefore obvious that the ion-binding capacity of a tissue would be closely related to its physiological state.[15, 16] Since ion exchange can occur with both organic and inorganic ions, it would seem probable that ion exchange activities of ground substance could be extended to include its interaction with organic ions as well as the inorganic.

Acidic mucopolysaccharides exist in the tissues as complexes with other naturally occurring substances An example of this may very well be the

some workers to conclude that heparin can bind histamine in the tissues [17, 18] Recently, Werle and Amann,[19] as well as Sanyal and West,[20] have demonstrated that heparin will bind definite amounts of histamine under standardized *in vitro* conditions In our laboratory Brinck-Johnsen[21] has found that under similar conditions this amine can be bound by other acidic mucopolysaccharides as well as heparin and, furthermore, that basic histamine releasers, such as compound 48/80* and polymyxin B, enhance the dissociation of these histamine-mucopolysaccharide complexes This would appear to be an ion exchange, since these basic histamine releasers also form complexes with heparin The fundamental importance of histamine has been discussed by Eyring and Dougherty in their theory of molecular mechanisms in inflammation and stress.[22]

Methods and Materials†

Complex formation The formation of heparin complexes with various drugs has been determined as follows the system consists of 120 μg of the dye toluidine blue (G Grubler and Co , Leipzig, Germany) in complex with 50 μg of heparin (117 μ/mg) in a series of tubes Graded amounts of the test substances were added to make a final volume of 6 0 cc , all substances having been dissolved in deionized water. The toluidine blue-heparin complex has an optimal absorption at 540 mμ in contrast to the optimal absorption of "free" toluidine blue at 630 mμ The dissociation of the toluidine blue-heparin complex in the presence of basic substances for which heparin has an affinity can therefore be determined spectrophotometrically The amount of free dye can be determined and the results plotted against the respective amounts of basic substance used The relative affinities of various substances for heparin can be compared

* Compound 48 80 (*p*-methoxy phenylethyl methylamine, a condensation product with formaldehyde) is an experimental product of Burroughs Wellcome & Co (U. S A) Inc , Tuckahoe, N Y

† The methods used in these experiments have been described in greater detail elsewhere [23-25]

STUDIES ON THE FUNCTIONAL INTERRELATIONSHIP OF FIBROBLASTS AND GROUND SUBSTANCE MUCOPOLYSACCHARIDES *

By R. D. Higginbotham
University of Utah Medical School, Salt Lake City, Utah

The distribution of loose connective tissue throughout the body as a "packing material" for blood vessels implies that it is an intermediary in the transport of metabolites to and from parenchymal cells, and may thus serve as a site for detoxification of endogenously produced noxious substances of the tissues (for example, histamine). These functions would indicate that the preservation of the physiological state in this tissue is necessary for the proper functioning of the parenchymal cells and, ultimately, of the organism as a whole. The maintenance of this physiological equilibrium is therefore dependent upon the interrelated activities of the loose connective tissue constituents

The fibroblasts are the major cellular constituents of the loose connective tissue and can secrete a number of the ground substance mucopolysaccharides [1] The ground substance, which has been described as the amorphous continuum separating cells, vessels, and fibers of the connective tissues, consists of such materials as proteins, salts, and water as well as of the mucopolysaccharides that characterize it [2] Thus the ground substance of the loose connective tissue with its complement of mucopolysaccharides constitutes the extracellular environment of the fibroblast. The synthesis, as well as the catabolism of mucopolysaccharides, would appear to be a function of these cells. These processes can be modified by various hormones [3] The interrelationship of fibroblasts and ground substance mucopolysaccharides can therefore be viewed as one aspect of the dynamic state of interaction between the cell and its environment This interaction of cells and their environment is a natural basis for the regulation of the physiological state of a tissue. In this regard, acidic mucopolysaccharides form acid-base complexes with various noxious substances that are subsequently ingested by fibroblasts of the loose connective tissue [4, 5] We have named this phenomenon micellophagosis and have postulated that it is a mechanism for the maintenance of the physiological state in the tissues [6, 7]

The acidic mucopolysaccharides can be briefly and generally described as macromolecular polymers of hexoseamine and hexuronic acid units that possess ionic properties arising from their uronic acid constituents that may be supplemented by varying amounts of esterification with sulfuric acid Specific information is available in reviews and communications concerning the respective structures, properties, and distributions of the acidic mucopolysaccharides of the ground substance [8-11] Ground substance may function

* ... -ported by Grant No DA 99 007 MD-13 from ... ashington, D C , and grants from Burroughs ... Y , and Chas Pfizer & Co , Inc , Brooklyn, N Y

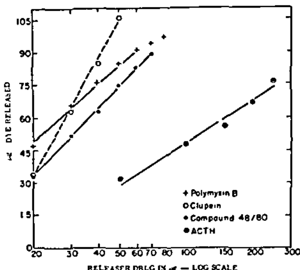


FIGURE 1 Comparison of the dye releasing activities of graded amounts of polymyxin B, compound 48/80, clupein, and ACTH Note that all substances complex with heparin, as shown by their release of toluidine blue

by Jacques.[26] The dye release occurs in a linear fashion relative to the log dose of releaser substances (FIGURE 1). The affinity of heparin for various drugs, relative to its affinity for toluidine blue, can thus be compared

A number of toxic drugs have been tested in this system, and it has been found that certain antibiotics (including polymyxin B, neomycin, and streptomycin), histamine releasers (such as compound 48/80 and stilbamidine), and various other substances, including the venom of Russell's viper, possess this ability[22, 27, 28] The relative affinities of heparin for representative basic substance of diverse chemical natures are shown in FIGURE 1 It is evident that this polysulfated mucopolysaccharide can react with a variety of substances Its affinities for compound 48 80[29] and for polymyxin B (a basic polypeptide)[30] are comparable to its affinity for the protamine substance clupein[31] Its interaction with the basic protein ACTH is much weaker

The demonstrated *in vitro* exchange of one cation (toluidine blue) for another, such as the toxic histamine releaser compound 48/80, would have a protective value *in vivo* For instance, heparin in complex with histamine would exchange cations as it formed a second complex with compound 48/80 and thereby reduce the active concentration of this latter substance Although the protection thus achieved would be gained at the expense of exposing the tissues to the released stores of tissue histamine, this exchange could be markedly beneficial, since compound 48 80 is many times more toxic than histamine[32] In this respect, the inability to exchange could be fatal These points will be considered further in this paper

Tissue sampling. CBA mice, 8 to 10 weeks old, were injected subcutaneously on the dorsal surface with 1 0 cc of air to form an air bubble in the loose connective tissue. Test substances, in a volume of 0.2 cc., were then injected into the bubble formed within this tissue. After sacrifice of the animal at an appropriate interval following injection, the connective tissue bubble was dissected free of the surrounding tissues and a section of it rapidly removed, spread on a glass microscope slide, air dried, and stained with May-Grunwald-Giemsa. Similar preparations were stained with azure A or toluidine blue for comparison Test substances, suspended in isotonic sterile saline, consisted of 500 µg of mucopolysaccharide either alone or as a mixture with 100 µg. of an appropriate basic substance per injection dose. A number of mice were prepared in this manner, and at least 3 mice were sacrificed at each interval for sampling.

Tolerance studies. To evaluate the effects of acidic mucopolysaccharide on the tolerance to mice of lethal doses of various noxious substances, both intraperitoneal and intravenous routes of drug administration have been used In the first case, animals were pretreated with a standard dose of heparin by the intraperitoneal route and challenged 30 minutes later with graded amounts of the toxic substance by the same route In the second case, a series of graded doses of the test mucopolysaccharide, each containing a standard lethal amount of a given challenge substance, was administered as a single intravenous injection The effects of the mucopolysaccharides on tolerance were determined on the basis of numbers of animals surviving 24 hours after challenge*

Acidic Mucopolysaccharides in Vitro

To investigate the potential *in vivo* ability of acidic mucopolysaccharides to act as ion exchangers for various organic substances, a simple *in vitro* model system consisting of a toluidine blue-heparin complex has been used to simulate a readily dissociable mucopolysaccharide complex of the tissues[23] Those substances for which heparin has an affinity can replace the cation toluidine blue in this complex and have therefore been designated as "releasers" The releaser-heparin complex, at optimal proportions, forms a precipitate, and the dissociated dye is freely dialyzable The equilibrium of this reaction would be governed by the relative concentrations of this cationic dye and the releaser substances, as well as by the dissociation constants of their respective heparin complexes These interactions have been considered in greater detail

* [illegible]

[illegible]

Acidic Mucopolysaccharides in Vivo

Fate in tissues. It has been previously observed that metachromatic granules appear in local fibroblasts of the loose connective tissue following the subcutaneous injection of heparin[5, 22] In FIGURE 2*a* fibroblasts of the loose connective tissue are shown with these metachromatic granules as they appear 2 hours after the injection of this mucopolysaccharide Heparitin sulfate (described by Meyer as a monosulfated heparin)[10] is also ingested by these cells (FIGURE 2*b*). Metachromatic granules can also be found in a few fibroblasts following the injection of chondroitin sulfate A (FIGURE 2*c*). However, they are not easily observed On the other hand, chondroitin sulfate B,[9] originally called beta heparin, is readily deposited as cytoplasmic granules by the fibroblasts (FIGURE 2*d*). The observations made regarding the fate of these mucopolysaccharides are summarized in TABLE 1. It is indicated that fibroblasts can ingest various types of these substances that temporarily are stored as granules in their cytoplasm before disposal, this is micellophagosis.[5] Fibroblasts thus participate in the catabolism of ground substance mucopolysaccharides as well as in their synthesis This process is not to be confused with phagocytosis, which includes the active cytoplasmic engulfment of *foreign* particulate material and is more ably performed by cell types occurring only in limited numbers in *normal* loose connective tissue.

Additional studies employing chemically sulfated preparations of hyaluronic acid and polysulfated chondroitin sulfate revealed that these substances appeared in the fibroblasts within two hours. However, cells that had ingested these chemically altered mucopolysaccharides appeared unable to dispose of these materials In marked contrast to the 24- and 48-hour persistence of the natural mucopolysaccharides, these substances persisted as granules in their cytoplasm for at least one month after injection Thus sulfated hyaluronic acid, which has been reported to be chemically similar to heparin,[24] is not as readily dealt with by cellular mechanisms as is the latter substance (FIGURE 2*e*). It is more toxic to fibroblast tissue cultures than equivalent amounts of heparin[24] and, in the present study, it initiated a marked

TABLE 1
TIME OF APPEARANCE AND PERSISTENCE OF TISSUE MUCOPOLYSACCHARIDES AS METACHROMATIC GRANULES IN FIBROBLASTS

Substance*	Intracytoplasmic granules	Granule persistence
Natural		
Hyaluronic acid	− (vacuoles)	—
Heparin	+ (2 hours)	24 to 48 hours
Heparitin sulfate	+ (2 hours)	24 to 48 hours
Chondroitin-SO_4 A	± (5–6 hours)	—
Chondroitin SO_4 B	+ (2 hours)	24 to 48 hours
Polysulfated		
Hyaluronic acid	+ (2 hours)	31 days
Chondroitin SO_4	+ (2 hours)	31 days

* Mucopolysaccharide (500 μg) was injected subcutaneously into mice At appropriate intervals thereafter, 3 mice in each group were sacrificed and tissue samples obtained from the area of injection

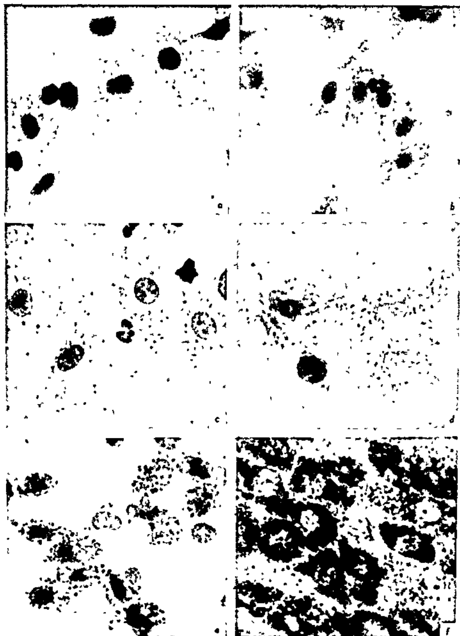


FIGURE 2 Air-dried spreads of loose connective tissue of the mouse, showing meta

and deposit it as rather large vesicular granules that often completely fill the cytoplasm of these cells (FIGURE 4d) and appear within about 2 hours after injection. Like heparin, these complexes seem to be digested within 24 to 48 hours.

Each of these basic substances, compound 48/80, polymyxin B, and clupein, can produce lethal intoxication in the mouse. The demonstrated fibroblastic sequestration of mast cell granules and of these heparin complexes can therefore be considered as evidence of a collaborative function of cells and acidic mucopolysaccharides in the maintenance of the physiological state in the tissues.

Acidic Mucopolysaccharides and Tolerance

Enhanced tolerance. The tolerance of mice to either compound 48/80[4] or polymyxin B[25] has been shown to be related directly to the concentration of administered heparin in the tissues. A quantitative dose response interrelationship of heparin and polymyxin B is shown in FIGURE 5. Mice given standard doses of heparin by the intraperitoneal route were separated into groups of 5 animals each and challenged 30 minutes later with increasing concentrations (5 µg/gm increments) of polymyxin B. Each of these titrations was performed at least twice. The results obtained were accumulated and plotted in terms of the LD_{50} doses of polymyxin B. The straight line was obtained by the method of least squares. These data indicate that increasing doses of heparin give increased tolerance to a given dose of the toxic agent and, with increasing doses of the latter, increased amounts of heparin

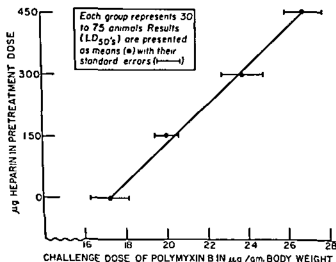


FIGURE 5. [illegible]

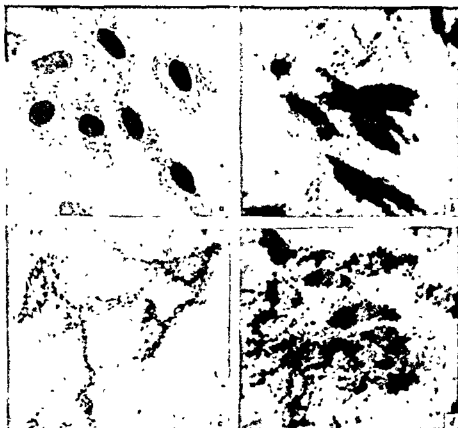
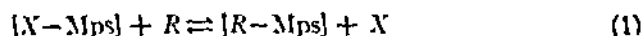


FIGURE 4 Air-dried spreads of loose connective tissue at noted intervals of time after

granules are often distinctly different from those of "free" heparin Cells filled with heparin in complex with the histamine releaser, compound 48/80 (FIGURE 4*b*), can be compared with the cells of a heparin control (FIGURE 4*a*) Granules of this heparin complex appear within 30 minutes after injection, in contrast to the two hours required for the appearance of granules in the control preparation Cells filled with this material more closely resemble a mast cell than a fibroblast[4] Fibroblasts also ingest a complex of polymyxin B-heparin (FIGURE 4*c*), and the granules are apparent within 30 to 60 minutes and are usually more numerous and slightly darker in color than those in the heparin control These cells will also ingest a complex of heparin and clupein

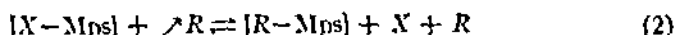
can be taken as evidence of the formation of drug-mucopolysaccharide complexes that are fairly stable *in vivo*. In this manner, the "free" or active concentration of an otherwise lethal dose of a drug is reduced to a level of measurable tolerance in the tissues of the animal (that is, 50 per cent survival).

On the basis of the foregoing studies, it has been considered that the natural capacity of the tissues to tolerate given amounts of such cationic substances as compound 48/80, polymyxin B, and clupein is related to the capacity of endogenous mucopolysaccharides in the tissue to form complexes with them. Tolerance can thus be schematically represented as the capacity of an easily dissociable mucopolysaccharide complex to form a second complex with the noxious substance:



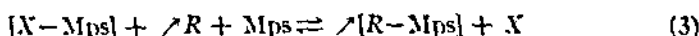
$$[X-\text{Mps}] + R \rightleftharpoons [R-\text{Mps}] + X \qquad (1)$$

in which X is any basic substance of the tissues, such as histamine, which can be replaced by a given dose of the toxic releaser substance R as the mucopolysaccharide Mps forms a second complex with the toxic drug. Toxicity would then be represented as an overwhelming of this capacity by an increased dose of the toxic substance which would enable more of it to remain free to interact at other and more vital sites in the tissues.



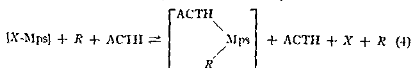
$$[X-\text{Mps}] + \nearrow R \rightleftharpoons [R-\text{Mps}] + X + R \qquad (2)$$

However, an *enhanced tolerance* to an increased amount of the toxic agent can be achieved by the administration of exogenous mucopolysaccharides (FIGURE 5, TABLE 2).



$$[X-\text{Mps}] + \nearrow R + \text{Mps} \rightleftharpoons \nearrow[R-\text{Mps}] + X \qquad (3)$$

Decreased tolerance This concept of acidic mucopolysaccharides in the maintenance of the physiological state in the tissues is analogous to a cation exchange reaction in which the mucopolysaccharide is the exchanger The capacity of an ion exchanger is a quantitative measure of its ability to exchange one substance for another and, during the course of its operation, this capacity would become smaller unless regenerated Thus, the changing and heterogeneous nature of the substances in and moving through the ground substance could modify the capacity of the endogenous mucopolysaccharides to perform this function The possibility has been examined that naturally occurring substances that can form complexes with heparin, such as ACTH, may decrease the capacity of the tissues to tolerate certain of the toxic drugs.[23] In this event, it would be expected that this reduced binding capacity of the tissues would result in a *reduced tolerance* in which a relatively larger amount of a given dose of a toxic substance would remain free to interact with more vital sites or activities in the tissues (SCHEME 2) and thereby initiate intoxication



$$[X\text{-Mps}] + R + \text{ACTH} \rightleftharpoons \begin{bmatrix} \text{ACTH} \searrow & \\ & \text{Mps} \\ R \nearrow & \end{bmatrix} + \text{ACTH} + X + R \qquad (4)$$

TABLE 2

COMPARISON OF THE EFFECTS OF VARIOUS ACIDIC MUCOPOLYSACCHARIDES ON THE TOXICITY OF CERTAIN NOXIOUS AGENTS IN MICE*

Drugs and challenge doses	Mucopolysaccharides			
	Heparin	Heparitin sulfate	Chondroitin sulfate A	Chondroitin sulfate B
	50 per cent protective doses (μg/gm)			
(1) Compound 48/80 (2 6 μg/gm)	0 36 (±0 02)	12 9 (±2 97)	none	none
(2) Polymyxin B (5 0 μg/gm)	0.56 (±0 01)	5.3 (±0 88)	none	5 4 (±1 55)
(3) Clupein (50 0 μg/gm)	7 13 (±0 09)	18 7 (±4 35)	19 0 (±4.0)	15 35 (±1 35)

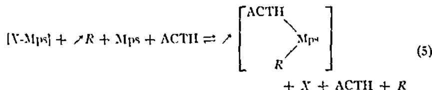
* Groups of mice, 10 animals in each, were injected intravenously with single doses of graded amounts of one of the test mucopolysaccharides Each dose contained a standard *lethal* amount of the respective challenge agent Results are presented as the 50 per cent protective dose of mucopolysaccharide with its standard error.

are required to achieve the same degree of tolerance. Related studies have shown that heparin can enhance the tolerance of mice to various other noxious substances with which it can form complexes[27] This tolerance therefore appears to be initially dependent on the direct interaction of heparin with the noxious agent rather than on an indirect effect of heparin in some detoxifying process in the tissues.

A qualitative and quantitative comparison of the respective abilities of various acidic mucopolysaccharides to form complexes with certain noxious basic drugs and thereby influence their toxicity is shown in TABLE 2. Groups of mice were injected intravenously with single doses of a series of graded amounts of one of the acidic mucopolysaccharides, each dose containing a standard lethal amount of one of the challenge agents The numbers of surviving animals in each of the challenged groups (10 animals in each) were recorded, and the amount of mucopolysaccharide necessary to protect 50 per cent of the mice was determined This procedure was repeated with each of the mucopolysaccharides versus each of the lethal drugs

Qualitatively, heparin and heparitin sulfate decreased the toxicity of all three challenge agents, whereas chondroitin sulfate A was effective only against clupein. However, chondroitin sulfate B was effective against both polymyxin B and clupein. Quantitatively, heparin was approximately 10 times more active than the other mucopolysaccharides with regard to the toxicities of compound 48/80 and polymyxin B, but only 2 to 3 times more active against the toxicity of clupein In general, their relative activities would appear to be: heparin > heparitin sulfate > chondroitin sulfate B > chondroitin sulfate A. It is evident that acidic mucopolysaccharides may differ both quantitatively and qualitatively in their abilities to reduce the toxicity of chemically diverse noxious substances. The reduction in toxicity of these basic drugs, when administered as complexes with the various acidic macromolecules,

Further studies performed to investigate these findings considered the possibility that, if ACTH were acting through the mechanism suggested above, it should also reduce heparin-enhanced tolerance to otherwise lethal doses of polymyxin B In this event, the *neutralized tolerance* would be schematically represented as a composite of SCHEMES 3 and 4.



$$[X\text{-Mps}] + \nearrow R + \text{Mps} + \text{ACTH} \rightleftharpoons \nearrow \left[\begin{array}{c} \text{ACTH} \\ \searrow \text{Mps} \\ \swarrow \\ R \end{array} \right] + X + \text{ACTH} + R \qquad (5)$$

A lethal dose of polymyxin B (5 0 μg./gm) was employed. This dose could be tolerated when administered in combination with a small dose of heparin (1 5 μg /gm). This combination was injected intravenously as a mixture with 25 μg /gm of the test substances in the same manner as before. In part B of TABLE 3 it is shown that ACTH also potentiated the toxicity of polymyxin B in this situation This demonstrates that this effect can be accomplished by a neutralization of the protective effect of heparin. Clupein, as well as toluidine blue, also reversed the protective effect of heparin in these mice challenged with otherwise lethal doses of polymyxin B It can be concluded from these experiments that the effects of acidic mucopolysaccharides on tolerance to noxious substances can be markedly influenced by other substances in the tissues that can compete with the toxic drugs for available groups on these acidic macromolecules

Discussion

The theory has been proposed that fibroblasts and mucopolysaccharides have a collaborative role in the preservation of the physiological state in the tissues Dorfman's group has clearly demonstrated that these macromolecules have a measurable rate of turnover in the tissues, and that this can be influenced by various hormones[4, 27] Turnover of acidic mucopolysaccharides can be visualized as consisting of their synthesis by fibroblasts, followed by their secretion into the ground substance from which they can be subsequently ingested by these same cells and then dealt with according to the metabolic capacity of the individual cell The physical and chemical states of the ground substance are therefore a reflection of both qualitative and quantitative aspects of these cellular activities This influence of the metabolic activities of the cell on the nature of the ground substance is an integral part of an interdependent relationship of cell and ground substance in which the latter, serving as the extracellular environment, influences the function of the cell This interaction of cells and environment is a natural basis for the regulation of the physiological state of this tissue

The polyanionic nature of the ground substance mucopolysaccharides is analogous to the essential molecular characteristics of cation exchange resins[14] Martin[28] has stated that the behavior of the ion-active groups of these latter

TABLE 3
EFFECTS OF ACTH AND OTHER BASIC DRUGS ON TOLERANCE OF MICE TO POLYMYXIN B

Challenged groups	Drugs added to challenge dose (25 μg /gm)	Number of survivors
A. Sublethal polymyxin dose*		
(1) Polymyxin B	Control	35/35
(2) Control	ACTH	10/10
Polymyxin B	ACTH	6/15
(3) Control	Clupein	10/10
Polymyxin B	Clupein	1/10
(4) Control	Toluidine blue	10/10
Polymyxin B	Toluidine blue	0/10
B Lethal polymyxin dose plus heparin†		
(1) Polymyxin B	Control	0/15
(2) Polymyxin-heparin	Control	15/15
(3) Polymyxin-heparin	ACTH	1/10
(4) Polymyxin-heparin	Clupein	0/10
(5) Polymyxin-heparin	Toluidine blue	0/10

* Intravenous injection of 3 5 μg polymyxin B/gm alone (1) or as a mixture with one of the test substances (2–4)
† Intravenous injection of 5 0 μg polymyxin B/gm (1) plus 1 5 μg heparin alone (2) or as a mixture with one of the basic test substances (3–5)

This possibility was evaluated by injecting groups of mice intravenously with single doses of graded amounts of ACTH, each dose containing a standard sublethal amount of polymyxin B.[23] It was found that this otherwise nonlethal dose of polymyxin B became progressively more toxic with increasing amounts of ACTH. This was interpreted to mean that the polymyxin tolerance of the tissues is related to the polymyxin-binding capacity of the endogenous mucopolysaccharides that could be reduced by ACTH Thereby more polymyxin remained free to interact with more vital sites in the tissues and thus induce lethal intoxication

The importance of ACTH as a hormone necessitated further study regarding the specificity of this ACTH effect TABLE 3 contains a summary of some of the experiments performed to evaluate this activity Groups of mice were challenged by the intravenous route with mixtures containing 25 μg of the respective test drugs gm body weight with the standard sublethal dose of polymyxin (3 5 μg gm) It can be seen that the dose of polymyxin is not lethal by itself, but becomes so when injected as a mixture with ACTH Similarly, a nonlethal dose of clupein will enhance the toxicity of polymyxin This shows that the ACTH effect on polymyxin toxicity is nonspecific and supports the idea that ACTH potentiates polymyxin toxicity by competing with this substance for the endogenous mucopolysaccharides of the tissues[24] This was further supported by the next result in which it is shown that the basic dye toluidine blue, which has a well-known affinity for heparin both *in vivo* and *in vitro*, will also enhance this effect

of a noxious character and represent a potential source of injury to the tissue. Turnover of this material is therefore essential to the continued normal functioning of the tissue as a whole. We have considered that the occasional and mild degranulation of mast cells with the subsequent fibroblastic ingestion of this material represents a salutary process whereby the stored granular material of the mast cell is made available to the more active metabolic processes of the fibroblasts.[6, 24] This disposition of stored material can prevent the accumulation in the tissues of excessive amounts of such substances as histamine which, in the event of injury to the tissues, can be released and further contribute to an already unphysiological condition.

Summary

These studies have considered a tissue phenomenon, termed micellophagosis, which is a collaborative role of fibroblasts and ground substance mucopolysaccharides in the maintenance of the physiological state of the loose connective tissue. The relationship between cells, mucopolysaccharides, and basic substances in this tissue are indicated diagrammatically in FIGURE 6. The fibroblastic ingestion of shed mast cell granules or foreign polysaccharides, as well as of tissue mucopolysaccharides, may function as a local means of detoxification. In the latter case, the acidic mucopolysaccharides may bind with basic substances (including amines, polypeptides, and proteins) released from injured cells, and the resulting complex may then be taken up by the fibroblast. If possible, these ingested materials will be digested. However, if the material is sufficiently altered or foreign to the cell, or the cell itself is metabolically deficient, such material may persist for a considerable period of time as stored granules. Cells containing such material are often morphologically similar to tissue mast cells (quasi-mast cells). The presence of both an acidic mucopoly-

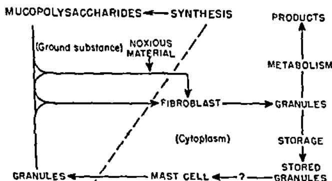


FIGURE 6. General scheme of the functional interrelationship of fibroblasts and acidic mucopolysaccharides of the ground substance. Reproduced by permission from the *Bulletin of the Reticuloendothelial Society*.[6]

substances is identical with that of those same groups attached to soluble molecular units. The ability of the acidic mucopolysaccharides to act *in vivo* as ion exchangers would be modified by the heterogeneous nature of the various substances in and moving through the ground substance. The equilibrium of interactions with various cationic substances would be governed initially by the relative concentrations of the cations concerned, as well as by the dissociation constants of their respective mucopolysaccharide complexes.

For theoretical purposes it has been considered that the tolerance of tissue for certain toxic histamine releaser substances is related to the ion exchange capacity of the endogenous mucopolysaccharides of the tissues[7, 23, 27] In this regard, a subliminal dose of these toxic agents is one in which the ion exchange capacity is not overwhelmed, the activity of the toxic substance being restricted by its formation of a complex with the acidic mucopolysaccharide The enhanced tolerance of heparin-treated mice for otherwise lethal doses of the histamine releasers compound 48/80 and polymyxin supports this idea and has been discussed[4, 5, 25] In the present study it was shown that other acidic mucopolysaccharides can also reduce the toxicity of these basic substances, but that they differ from heparin both quantitatively and qualitatively in this effect

Although the ion exchange reactions of the mucopolysaccharides would be of a reversible nature, the formation of less easily dissociable complexes would tend to reduce the ion exchange capacity of these macromolecules. The interaction of the mucopolysaccharides with a given amount of one basic substance could reduce their ability to form complexes with a second basic substance In the event that the second substance is toxic, the capacity of an animal to tolerate this drug would be reduced in proportion to the reduction in the capacity of the mucopolysaccharides to exchange ACTH and other basic substances that form complexes with heparin will potentiate the toxicity of polymyxin B This has been interpreted to mean that ACTH can compete with polymyxin B for the available ionic groups on the *endogenous* mucopolysaccharides[23] It was demonstrated that ACTH, toluidine blue, and clupein can neutralize the protective effect of *administered* heparin on polymyxin B toxicity. The influence of acidic mucopolysaccharides on the physiological state in the tissues is therefore related to the physical and chemical states of these macromolecules, as well as their concentrations The tendency for these substances to reach a relatively stable (that is, less reversible) state necessitates a physiological means for regeneration of the ion exchange capacity of the ground substance This could be accomplished directly by local increases of inorganic cations, such as sodium, and indirectly by the renewal of the mucopolysaccharides by the cells

Metabolism of the ground substance mucopolysaccharides is a natural and necessary cellular event The inability of cells to perform this process after ingestion of these substances inevitably leads to their storage within the cell, accompanied by the storage of whatever other substances these acidic macromolecules may have bound The presence of such material in a cell implies a physical reduction in its capacity to perform its essential cellular activities, in addition, those substances bound to the acidic mucopolysaccharides may be

[illegible] Univ Utah Salt Lake City, Utah
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38 MARTIN, G J 1955 Ion Exchange and Adsorption Agents in Medicine Little Brown & Co Boston, Mass

appear to be analogous to that of ion exchange resins in which various inorganic or organic cationic substances can be bound or released under the appropriate conditions. This is a dynamic process in which the exchangers are regenerated by synthetic and catabolic activities of the cells and interact, according to their specificities and capacities, with the various substances in transit between the plasma and cells of the interstitium and parenchyme.

Acknowledgments

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MORPHOLOGY, CHEMISTRY, AND FUNCTION OF MAST CELLS*

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Introduction

Our appreciation of the mast cell as a distinct cellular entity is now just eighty years old. It was initiated by Ehrlich's discovery[1] of a group of cells exhibiting a strong basophilic metachromasia. In doing so he differentiated these from the larger group of mononuclear cells previously recognized in connective tissues. Since this time a voluminous literature concerning these cells has accumulated[2-6] The large mass of data, which unfortunately contains some misinformation, is confusing A part of this confusion can be obviated by a consideration of the following. Cells of like nature have fundamental similarities, but they also have subtle differences The mast cell, which appears to be present in practically every vertebrate animal thus far examined, is such a cell type We must recognize at the same time both the broad similarities and the important differences between mast cells of various species. The systematic examination of the mast cells of a single species provides the beginning of the unraveling of its mysteries What follows is some of the information thus far at hand on the mast cells of rats

Morphology

Shape, size, and density Mast cells in various sites in the rat have a polymorphous or an oval form The polymorphous form is seen adjacent to blood vessels and in the connective tissue. It appears oval in the mesentery Free cells in the peritoneal fluid, when isolated, are round. The diameter of free cells averages 13 4 μ. The volume as measured by packing a known number of cells averages 1 32 $\pm$ 0.2 $\times 10^{-6}$ cu mm per cell A similar value is obtained by calculation from the mean diameter of the free cell or by estimates of volume made *in situ* In some sites such as the dermis of the skin the cells may appear smaller following shedding of their granules When isolated from the peritoneal fluid the cells appear to have a specific gravity that lies between 1 088 and 1 100. These values were derived from measurements made in concentrated albumin Previously we had found that in a concentrated sucrose medium the cells would sediment in a density in excess of 1 26[7] This appears now to have been due to the fact that the cells shrink in the very hypertonic sucrose

Staining properties. Characteristically, mast cells stain with any basic dye such as toluidine blue, and they exhibit characteristic metachromasia The staining appears to us to be entirely resident in the cell granules The granules do not stain with hematoxylin under most circumstances, but occasionally

one can see pale blue staining with this dye. Their nuclei are, in general, round and resemble slightly those of plasma cells or histiocytes. Thus, in hematoxylin-eosin preparations the cells are not usually differentiated from other mononuclear connective tissue elements They do stain with the Schiff reagent following periodic acid oxidation.

Electron microscopic characteristics. In the electron microscope the granules are dense after osmic acid fixation. They have round, oval, or irregular forms; the latter is probably an altered form. We[8] have been unable to discern any fine structure within them. There is a less dense intergranular material surrounding and connecting the adjacent granules We have not yet seen any mitochrondrial structures of characteristic configuration. Occasionally a thin dense line at the perimeter of the cell is visualized, suggesting a tenuous cell membrane

Chemistry

Heparin. Lison[9] suggested that the metachromasia exhibited by the basic dyes under the appropriate conditions was indicative of the presence of compounds containing ester sulfate radicals Holmgren and Wilander[10] connected the presence of considerable sulfate in heparin and the metachromasia of mast cells Together with Jorpes[11] these investigators showed that tissues rich in mast cells also contained much heparin, Ehrlich and others confirmed this We have now obtained direct evidence that a material with the properties of heparin is present in mast cells and have estimated its concentration. By modifying the method of Padawar and Gordon[12] to provide a larger yield and purer (90 to 100 per cent) preparation of mast cells, a sufficient quantity could be obtained for analysis [13] The analysis of one preparation is given in TABLE 1. The material isolated had the appropriate metachromasia and appropriate mobility on chromatography It had anticoagulant activity and contained hexosamine The quantity of material present in the cell estimated from the several available parameters ranged between 2.7 and 4 6 per cent of the cell volume.

Histamine and 5-hydroxytryptamine Riley[4] and Fulton *et al* [5] provided evidence indicating the probable presence in and release of histamine from

TABLE 1
HEPARIN IN ISOLATED RAT MAST CELLS

[illegible] Prepared [illegible]

[illegible] digestion under dialysis [illegible]

[illegible] color appropriate

Hexosamine content glucosamine equivalent 1 08 per cent of cell volume Modified Morgan and Elson procedure Calculated to be equivalent approximately to 4 6 per cent of volume of mast cell

Table 2

Evidence that Histamine and 5 Hydroxytryptamine are Associated Mainly with Mast Cells in Rat Skin

Skin region	Mast cells No./h.p.f.*	Histamine	5-HT†
		µg/gr.	µg/gr
Feet, areolar subcutaneous	33.1	259	10.7
Feet, dermis	6.4	74	2.1
Back, areolar subcutaneous	5.1	36	1.8
Back, dermis	3.0	20	1.5

* High power microscopic field
† 5-Hydroxytryptamine

Table 3

Characteristics and Quantities of Histamine and 5-Hydroxytryptamine in Isolated Rat Mast Cells

	Histamine		5-Hydroxytryptamine	
	Synthetic	Mast cell	Synthetic	Mast cell
Pharmacologic assay				
Guinea pig ileum	+	11.2 µg/cu mm	+	+
Rat colon	−	−	+	0.6 µg/cu mm
Chromatographic mobility (Rf)				
Butanol-acetic acid-water	0.16	0.08–0.16	0.44	0.42
Color reactions				
Diazotized sulfanilamide	Red-orange	Red-orange	Violet	Violet
Dimethylaminobenzaldehyde	−	−	Violet-> blue	Violet-> blue
Fluorescence with HCHO			Yellow	Yellow

mast cells. We have found that 5-hydroxytryptamine is present in rat mast cells.[13] The association between mast cells and histamine and between mast cells and serotonin in the cutaneous and subcutaneous tissues of the rat is shown in table 2. From isolated purified mast cells we have been able to extract and to characterize these two substances as shown in table 3. As can be seen there, the concentration of histamine in the isolated mast cells is *11 µg/cu mm of cells, or 1.1 per cent of the cell volume. The concentration* of 5-hydroxytryptamine is approximately one twentieth of this or 0.06 per cent of the cell volume. Schayer[14] has provided evidence indicating that histadine decarboxylase is associated with the mast cell. We have demon-

Esterase with properties resembling chymotrypsin. Gomori[16] described the fact that mast cells are demonstrated selectively when tissue is incubated with chloroacetyl-2-hydroxy-3-naphthoic acid amlid under the appropriate histo-

Table 4

Properties of a Hydrolytic Enzyme in Mast Cells with Substrate Requirements Resembling Chymotrypsin

	Mast cells	Chymotrypsin
Substrates		
Chloroacetyl-2 hydroxy-3 naphthoic acid anilid (CANAS)	+	+
Acetyl tryptophane ethyl ester (ATrEE)	+	+
Acetyl tyrosine ethyl ester	+	+
Acetyl phenylalanine ethyl ester	+	+
Tosyl arginine methyl ester	−	−
pH Optima	8.4	8.0
Inhibition		
Di isopropyl fluorophosphate	70% at 1 × 10^{-4} M	60% at 1 × 10^{-4} M
Competitive between CANAS and ATrEE	+	not tried

chemical conditions. We have found (table 4) that this enzyme has properties resembling chymotrypsin.[17] If the enzyme has a similar activity to weight ratio as chymotrypsin, then its concentration in the cell is of the order of 1 per cent of the cell volume.

Absence of discernible succinic dehydrogenase and other mitochondrial enzymatic properties. We have thus far been unable to demonstrate succinic dehydrogenase activity in the mast cell of the rat, nor have we been able to demonstrate the characteristic color reaction of Janus green B. These facts suggest that there are only small amounts, if any, of the usual mitochondrial enzyme systems.*

The structure of mast cell granules. Several years ago we[8, 18] attempted to isolate mast-cell granules from the subcutaneous tissues of the rat. In the course of the experiment we found that the granules began immediately to swell following comminution of the tissue. During this and the subsequent process of purification the granules lost their characteristic capacity to stain [illegible]

this stage they are associated with a certain amount of connective tissue debris, a large part of which can be removed by washing in isotonic sodium citrate. These granule residues (figure 1) are about the size of blood platelets. They contain little, if any, hexosamine and no demonstrable heparin, histamine, or serotonin. Their size and, to some extent, their form induced us to compare their capacity to accelerate the clotting of recalcified platelet-poor citrated rat plasma with the known activity of blood platelets and also with similarly prepared rat liver mitochondria. The average behavior of the several different types of particles is recorded in figure 2. Here can be seen

* J. Padawar (personal communication) states that with nitrotetrazolium as histochemical indicator instead of neotetrazolium, which we used, succinic dehydrogenase activity is demonstrable in mast cells in rats.



FIGURE 1 Mast cell granule "ghosts" isolated from rat subcutaneous tissue in sucrose and washed with sodium citrate Giemsa stain ×2500

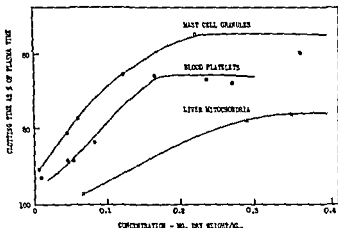


FIGURE 2 Thromboplastic activity of rat blood platelets, isolated mast cell granule "ghosts," and liver mitochondria

the usual behavior of blood platelets shown in terms of their capacity to reduce the clotting time of platelet-poor rat plasma. It can be seen that the mast cell granule "ghosts" prepared in sucrose and washed with citrate behaved in a manner similar to the platelets, but on a per-unit dry-weight basis they were more active Liver mitochondria were substantially less active This is presumptive evidence for participation of a part of the mast cell granule in tissue thromboplastic activity This "ghost" must also contribute to the local tissue in the response to injury

The evidence indicates that the cell granule is an organized complex structure One of its principal ingredients is heparin, another ingredient is probably histamine, which is present in an amount sufficient to account for a large proportion of the cation necessary to the presence of the strongly anionic heparin.[7] Histamine disappears from tissues simultaneously with the spill of mast cell granules Heparin and histamine are probably not present in a simple salt combination. The mast cell granule "ghost" may be a sort of matrix in which heparin and histamine are held Disruption of the matrix would then be required for histamine release Consistent with this notion we have observed that substantial amounts of 48/80* will not "displace" the histamine from isolated mast cells, but will release it from mast cells *in situ*. The chymotrypsinlike esterase may be present in the mast cell granule. The functional interactions of the enzyme are still obscure Previously we have reported[19] that chymotrypsin is capable of hydrolyzing peptides associated with such acidic mucopolysaccharides as chondroitin sulfate. It is reasonable, therefore, to suspect that this enzyme may operate in this fashion *in situ* We have no clear evidence thus far for this

5-Hydroxytryptamine may not be in the mast cell granule complex We have found that following reserpine administered *in vivo* the concentration of

* A chemical compound with the capacity to liberate histamine

5-hydroxytryptamine in isolated peritoneal mast cells is reduced by about 50 per cent without alteration of either the cell numbers, their morphology, or their histamine concentration. It may be that this material is in the intergranular substance. Another substance capable of disappearing without gross morphologic alteration is the pyridoxal-5-phosphate component of the 5-hydroxytryptophane decarboxylase system

Functional Properties of Mast Cells

The response to mechanical and chemical stimulation Mechanical manipulation, such as pinching the skin or administering a saline injection, will produce some spillage of mast cell granules. The effects of this are easily observed in the passage of Evans blue from the circulating blood into the local site Certain chemical substances, in particular 48/80, ovomucoid, dextran, and some yet-unidentified substance in testicular extract[20, 21] will produce in sufficient doses given intravenously, locally, or applied to surviving tissue *in vitro* mast cell disruption More chemical stimulators of mast cell granule release remain to be identified in animal tissues These will probably be more interesting than those substances thus far discovered.

Adrenal corticoids, cortisone, and hydroxycortisone, insofar as we can find, do not alter the numbers, morphology, or histamine content of mast cells when given for ten days in doses sufficient to cause thymus and adrenal involution and also body weight loss These indices of alteration may not be sufficiently sensitive, since Schayer[14] has observed a reduction in histamine formation induced by these compounds.

When mast cell granule release is produced by one of the several situations or agents mentioned above there is a simultaneous release of histamine and activation of 5-hydroxytryptamine.[20, 21] These operate locally upon the skin vessels in the rat to produce increased capillary permeability, which can be shown by the leakage of Evans blue from the circulation It is possible that the histamine that disappears rapidly from the local site can stimulate the pituitary to secrete adrenocorticotropic hormone (ACTH). The release of histamine from certain local sites provides the possibility of a "feedback" mechanism. Thus peripheral or visceral stimulation may (and a major injury to a part almost certainly can) activate the pituitary-adrenal cortex system via histamine[22]

Following the administration of large doses of ovomucoid we have not yet been able to find evidence of prolonged blood coagulation or activation of the lipemia-clearing factor, which one might expect from release of heparin into the circulation Riley and his coworkers[21] have reported that there is no reduction in tissue heparin following similar treatment of rats with 48 80 This evidence is consistent with the notion that the heparin interacts locally with some tissue elements present What it may interact with is not yet evident.

Conclusions

We can now state with reasonable assurance the following things about rat mast cells they contain about 3 to 4 per cent heparin, about 1 per cent histamine and, perhaps, 1 per cent of a hydrolytic enzyme There, together with

cytoplasm are probably 5-hydroxytryptamine, at least the pyridoxal-5-phosphate part of the 5-hydroxytryptophane decarboxylase, and other yet-undetermined substances When released, the substances interact in an organized fashion with local, some with distant, tissue components Mast cells thus can be classed both with stimulus receptor and secretory cells. Several chapters on the mast cell are partially written These need to be finished and the keys to the remaining chapters discovered

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MEASUREMENT OF THE FUNCTION OF THE RETICULOENDOTHELIUM

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Introduction

The identification of the reticuloendothelial system (RES) as such was made in 1913 by Aschoff and Kiyono.[1] Since that time constant attempts have been made to determine the functions of this system, as well as how to measure them. Great difficulties stemming from the very nature of the system have been apparent from the beginning. It is only recently that some of these difficulties have been solved through the introduction of physical methods of measurement

Problems Involved in Measuring RES Function

Delineation of RES A primary difficulty lies in the fact that there is no consensus as to all the types of cells that should be included within the system Investigators in this field use three major criteria for delineating this system The first group relies on morphological identification, using histological evidence and differential staining techniques The second school utilizes embryonal factors, and tends to include those cells that derive from primitive mesenchyme. The third group is more physiologically oriented and prefers to use more functional standards Many investigators utilize intermediate criteria Although all workers in the field always include such elements as macrophages, some violently debate the question whether eosinophils, mast cells, lymphocytes, and plasma cells should be included

Rather than become involved in this classic dispute, I shall rely upon the suggestion given by Chester Hyman of the University of Southern California at Los Angeles, who replied to the question of which cells should be included in the RES "Like the elephant in the ancient Hindu fable, the reticuloendothelial system has been certainly identified and defined by many people as many different things In Carl Sandburg's version of the Hindu classic, the men of different opinion ' did not put up any arguments. They did not throw anything in each other's faces Three men saw the elephant three ways and let it go at that ' "

[illegible] nature of the RES Added difficulties in measuring the [illegible] m [illegible] nd [illegible] m, thus precluding one classic method of measurement that involves the use of extirpation. The inability to remove a significant segment of the system also eliminates methods of measurement that involve chemical analysis of cellular components and products

The problem becomes considerably more complex when one considers that various cell types within the system have different functions As a matter of

fact, even similar cells such as macrophages, when located in different organs, appear to have somewhat dissimilar functions Thus there is evidence that liver RE cells have primarily a metabolic function, while those of the spleen appear to be more involved in antibody formation. Further, there is pre- [illegible]

stimulus, relatively undifferentiated cells can be stimulated to become RE cells, whereas under normal circumstances they would have developed into other elements.

Whether the new RE cells produced in response to a stimulus can perform all the functions of the regular RE elements is not known. Furthermore, the ability of fixed cells to become mobile and of mobile cells to become fixed leaves us with a system inordinately difficult to measure

Known and Suspected Functions

It is axiomatic that one must have at least some idea of a function before methods can be devised to measure it Among those functions of the RES which are known or suggested are· (1) phagocytosis of electronegatively charged particles, ranging in size from proteins to erythrocytes, (2) intracellular destruction or metabolism of phagocytized substances, (3) antibody formation—to the extent that phagocytosis appears to be one of the initial requisites in antibody production (if plasma cells and lymphocytes are included in the definition of the system, the role of the RES in antibody formation becomes further extended), (4) other aspects of host defense, such as the role of the RES in the host-tumor equilibrium and allergic phenomena, (5) detoxification of substances such as alcohol and barbiturates, (6) normal metabolic pathway for certain lipids, such as cholesterol; and (7) hematopoiesis.

A list of other suspected functions is too long to be catalogued here.

Early Attempts at Measurement

The first attempt to measure RES function relied upon histological techniques. Because the RE cells had a particular affinity for certain vital dyes, staining techniques became the first obvious method to use An extension of this procedure relied upon the ability of the RE cells specifically to sequester [illegible]

titative. Furthermore, as with most histological methods, it has the disadvantage of giving the investigator a picture of a single point in time in a single animal, and the extrapolation back to an *in vivo* and dynamic state is always difficult Finally, because of the diffuse nature of the RES, the histological examination of an animal becomes a tremendous chore in that it involves the examination of most of the tissues throughout the body.

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engorge RE cells with particles in order to produce blockade. The shortcomings of dyes lie in the fact that many are not pure chemicals, and one production batch can differ from the next. Furthermore, since most of them are not completely colloidal, the soluble fraction produces complications, since it is frequently cytotoxic. Different cytotoxic agents can induce a primary depression of RES function, which is frequently followed by a secondary compensatory hyperfunction that yields confusing results.

In addition, many of these substances have heterogeneous particle sizes, resulting in further error and in discrepancies between one batch of test substance and the next.

India ink is another colloidal substance that has enjoyed considerable use. Biozzi *et al.*[2] showed that the shellac used in making India ink (the amount and type will vary from one producer to the next) is toxic, and the net result is to produce intravascular aggregates, resulting in microemboli, particularly in the pulmonary circulation.

More Recent Attempts at RES Measurement

One of the most important steps in attempting to make consistent definitive and quantitative measurements of the RES was taken when the International Society for Research on the RES was founded in 1954. This society, which has a world-wide membership, has become, through its symposia, its regional and international meetings, and its journal, a vehicle for the exchange of information and the dissemination of data leading to the development of better and more precise methods of measurement with certain universally accepted criteria.

The current emphasis on developing methods of measurement of phagocytosis relies upon the use of inert colloids. In order to meet the criteria now generally accepted, these colloids, in addition to being inert, must have a uniform particle size (not only in the syringe, but also in the blood). They must not aggregate in the blood, and they must be susceptible of chemical or physical measurement in relatively small concentrations. Among the colloids now in use are carbon, metals, insoluble metallic salts, and plastic spherules. When such colloids are injected intravenously, their disappearance from the blood should follow an exponential function (FIGURE 1).

The intravascular half life of these colloids is easily calculated from such a curve. A more sophisticated mathematical approach to the analysis of such a curve may be had by using the formula suggested by Biozzi *et al.*[2] $C = C_0 10^{-KT}$.

Many investigators rely to a great extent on the use of colloidal carbon.* This carbon is stabilized in colloidal suspension by fish glue and gelatin, and has served very satisfactorily for many studies of phagocytosis. However, our studies have shown that the Gunther-Wagner type of carbon still leaves

* Obtained from Gunther-Wagner Pelikan Werke, Hannover, Germany.

much to be desired. An analysis of methods of producing this carbon shows that certain unavoidable errors are inherent in the method of production As this carbon is produced by incomplete combustion of hydrocarbons, there are, in addition to discrete particles, a significantly large number that are joined together (FIGURE 2).

Whereas very large particles of carbon can be sedimented out by differential centrifugation, the final product still presents a spectrum of particle sizes

In order to circumvent this problem, we are utilizing polysterene spheres made in such a way as to obtain a range of standard particle sizes, beginning at several hundred angstroms, with a maximum deviation in each group of 5 per cent. In order to tag these spheres a technique can be used that will swell their surfaces temporarily, giving rise to interstices on the surfaces Into these interstices one can introduce metallic atoms These atoms should have a high cross section for neutrons, so that bombardment in a moderate flux (10^8 neutrons) from a sealed-tube accelerator will induce radioactivity in the metal atoms but leave the polysterene virtually undamaged. Carbon can also be made in such a way as to obtain a range of standard particle sizes To do this one begins with the production of plastic spherules All elements other than carbon are driven off by pyrolysis, leaving a pure carbon spherule of definitive particle size The carbons can then be treated with a variety of chemical or physical techniques, resulting in a surface that can selectively adsorb various products This opens a whole new avenue of techniques for study of other RE functions, these I shall mention presently These homogeneous particles must then be suspended in a medium that maintains the particles as a true colloidal suspension It is imperative that, when the colloid

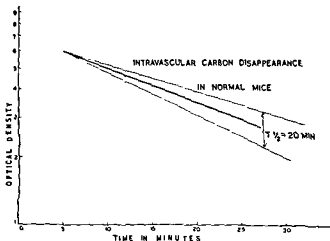


FIGURE 1 Exponential disappearance of intravenously injected carbon colloid The heavy line is the mean value of 25 mice, and the flanking light lines show the standard deviation From this curve it can be calculated that 50 per cent of the carbon disappears from the circulation in 20 minutes



FIGURE 2 Electron micrograph of the colloidal carbon that is in general use for RES testing This carbon is obtained by incomplete combustion of hydrocarbon in a limited oxygen atmosphere ×100,000

is injected intravenously, aggregation will not occur as a result of the altered physical-chemical conditions due to the presence of blood proteins and electrolytes The use of appropriate colloids and the calculation of the disappearance velocity from the blood can give considerable important data on phagocytosis by the RES

In the same general area of phagocytosis is a technique wherein radioactively tagged bacteria are injected and their uptake by RE cells studied This technique is useful in studying the ability of the RES not only to take up bacteria but also to destroy them Accordingly, when the RES is damaged,

as by radiation, phagocytosis is not significantly altered, but the ability of the RE cells to destroy the ingested bacteria is seriously impaired, conse-

gested bacteria

Another variation of this technique has recently been proposed by Benacerraf *et al*[3] They use a partially denatured serum protein fraction labeled with I^{131}. The protein is phagocytized and, as it is metabolized within the cells, iodine escapes back into the circulation The measurement of the rate of reappearance of iodine is thought to give an index of RES metabolic function. However, several problems exist with the use of iodine, including the role of liver iodase and thyroid uptake and urinary excretion. In order to circumvent these problems we are working with a method wherein we adsorb to our carbon colloids molecules tagged with radioactive elements that are not susceptible to metabolic interference in the body. In the case of adsorbed proteins, this technique demands that adsorption to the carbon should not be so great as to denature the proteins and yet be sufficiently strong to prevent desorption in the circulation

We are using another modification of the same approach in order to measure antibody formation as a function of the RES activity We accordingly

made in order to obtain an index of the role of the system in antibody formation.

Another method used to evaluate RES function capitalizes upon a method initially suggested by Rous and Beard[4] and more recently refined by St George *et al.*[5] Magnetic iron colloid is injected intravenously and is phagocytized by the RES. An organ such as the liver is forced through the mesh of a silk screen, resulting in a mixture of free RE and parenchymal cells If a strong magnetic field is placed at right angles to the container, the parenchymal cells will sink to the bottom, and the iron-containing RE cells will aggregate along the lines of magnetic force. This permits the isolation of a relatively pure group of RE cells. These cells cannot be considered completely normal, as they contain phagocytized iron colloid, but the technique is most useful and has been utilized by Friedman *et al*[6] in determining that the RE cells are significantly involved in the uptake of exogenous C^{14}-labeled cholesterol. Such a method is obviously quite effective in measuring uptake as well as metabolic functions of labeled phagocytized material

A useful method to determine some other functions of RE cells is to blockade or stimulate the RES "Blockade" is at best a relative rather than an absolute term, it indicates that cells capable of phagocytosis have phagocytized virtually all the material that they are capable of ingesting The term "blockade" must, however, be used with caution for, as determined by Biozzi *et al*,[7] the RES has a preferential affinity for certain colloids Thus,



FIGURE 2 Electron micrograph of the colloidal carbon that is in general use for RES testing This carbon is obtained by incomplete combustion of hydrocarbon in a limited oxygen atmosphere ×100,000

is injected intravenously, aggregation will not occur as a result of the altered physical-chemical conditions due to the presence of blood proteins and elec- [illegible]

In the same general area of phagocytosis is a technique wherein radioactively tagged bacteria are injected and their uptake by RE cells studied This technique is useful in studying the ability of the RES not only to take up bacteria but also to destroy them Accordingly, when the RES is damaged,

hyperlipemic fraction from the circulating blood, conversely, stimulation of the RES can accelerate it[8]

A similar blockading and stimulating technique can point up the role that the RES plays in the detoxification of barbiturates, alcohol, and other substances, quantitation of this function can be obtained by correlating the degree of blockade or stimulation in terms of the pharmacological effect of the substances studied[9] Similar techniques can demonstrate the role that the RES plays in the handling of bacterial endotoxins and exotoxins.[10]

Finally, current studies in our laboratory supported by the Damon Runyon Memorial Fund for Cancer Research, Inc., New York, N. Y, have indicated that blockade and stimulation of the RES appear to have a significant relationship to the percentage of "take" in certain transplanted tumors, in their rate of growth, and in metastasis One very useful agent in this study, which has an inhibitory as well as a stimulatory effect upon the RES, is zymosan Although most investigators are studying this agent in its role in the properdin system, we are studying its effect upon the RES The correlation of the RES effects of this substance with the growth or inhibition of tumor, as well as with infections, makes it a most intriguing area in which to use quantitative methods Further data on zymosan, its fractionation, and its effect on the RES is to be presented at the Third International Symposium on the Reticuloendothelial System (August 1958, in Italy)[11]

Phase-Contrast Time-Lapse Microcinematography

Another method of measurement that is useful in studying individual RE cells *in vitro* is the use of phase-contrast time-lapse microcinematography The use of such techniques in the hands of such investigators as A Policard from the Centre D'Études Recherches des Charbonnages de France in Paris and G. Voisin from the Hôpital Saint-Antoine, also in Paris, has given considerable data on the mechanisms involved in phagocytosis Other interesting phenomena can be correlated with this technique, such as the incredible cytotoxicity of a presumably inert silica particle as opposed to a variety of other colloids

In Vivo Depression or Destruction of the RES

An important technique—if it could be adequately evolved—would be the development of a colloidal particle that would be lethal to RE cells It would be assumed that, after such a colloidal particle had destroyed an RE cell, it would be released, rephagocytized and, in turn, destroy another RE cell The success of such a procedure depends upon the ability of the particle to kill RE cells more rapidly than they can be regenerated In our laboratory, S^{35}-labeled colloids could not destroy the RES because regeneration always exceeded the killing rate A more recent experiment is currently being carried out at Stanford University, Stanford, Calif by J G Pool, who is using tritiated polystyrene spherules This investigator uses material that delivers more than 250 mc per rat, but thus far she has been unable to destroy the RES

Chemical methods to depress the RES, using substances such as cortisone[12]



FIGURE 3 Radioautograph of mouse liver, showing characteristic RES localization of S^{35} labeled protein adsorbed to carbon colloids

if one injects two different colloids simultaneously, the one may be taken up first, to the virtual exclusion of the other Once the first colloid has been ingested, the second is then phagocytized It is therefore open to question whether cells that are "blockaded" with one colloid may not still be able to take up amounts of another colloid for which the RES has a high affinity Furthermore, some RE cells do not seem to take up colloid even if it is present in the circulation for a protracted period This may be due to the fact that not all the vasculature is patent, thus precluding the impingement of colloid upon certain RE cells In addition, the regenerative rate of RE cells is so tremendous that so-called complete blockade is at best short-lived

This blocking technique, although it is relative rather than absolute in nature, is nevertheless useful in evaluating certain functions For instance, if the hyperlipemic fraction of sera of a cholesterol-fed animal is injected intravenously into a second animal, the rate of disappearance of the cholesterol from the recipient animal can be plotted exponentially Blockade of the RES by an inert colloid can retard significantly the disappearance of the

Part III. Cytology and Etiology

CYTOLOGY OF THE STERNBERG-REED CELL AS REVEALED BY THE ELECTRON MICROSCOPE*

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Although Hodgkin's disease may have been described as early as 1661, Thomas Hodgkin's report in 1832[1] of several cases involving lymph node enlargement, cachexia, and fatal termination marks the beginning of many years of study of the disease that now bears his name. Forty years passed before cytologists reported the presence of giant cells in Hodgkin's disease[2] In the next three decades many investigators: Greenfield,[3] Dreschfeld,[4] Goldmann,[5] Dietrich,[6] and Sternberg[7] verified Langhans' original observation of giant cells in Hodgkin's disease[2] The complete description of these cells by Dorothy Reed[8] in 1902 has won her lasting fame She wrote: "The large giant cells vary from the size of two or three red blood cells to cells twenty times this size. The nucleus is always large in proportion to the size of the cell It may be single or multiple If single it is usually round or bean-shaped and irregularly dented nuclei are common . . . The chromatin network is prominent in these nuclei and one or more large nucleoli are always present. . . . The protoplasm is usually homogeneous, may appear granular, show vacuolization or contain fat or pigment granules Cells having bizarre and irregular nuclei are found in the oldest growths"[8]

In the fifty-six years since these observations were made scientists have invented and perfected a new tool, the electron microscope, for visualizing the smallest of structures, even those approaching molecular dimensions Although descriptions of electron micrographs of the Sternberg-Reed cell have been reported,[9-11] it is of interest to re-examine Dorothy Reed's description of the cell characteristic of Hodgkin's disease and to compare her description with what is seen in the electron microscope

Inasmuch as other contributors to this monograph have presented illustrations of the Sternberg-Reed cell as seen in the light and phase microscopes it is convenient to proceed directly to the electron microscopy of the cell FIGURE 1 is an electron micrograph illustrating the general appearance of lymph-node sections from patients with Hodgkin's disease The predominant [illegible]

This micrograph and those that follow were obtained by fixing the tissues immediately upon biopsy in 1 per cent buffered osmium tetroxide, dehydrating

* The work reported in this paper was supported in part by grants from the Dorothy H and Lewis Rosenstiel Foundation, New York, N Y, and the Andre Crotti Cancer Research Fund of the Ohio State University, Columbus, Ohio

and chlorpromazine,[13] are effective, but they involve too many other physiological parameters.

Conclusion

Because of the diffuse, diverse, and dynamic nature of the RES, all techniques aimed at physiological measurement must, perforce, be *in vivo* and indirect. However, it is to be hoped that the use of physical methods will permit an increasing development of quantitative techniques to evaluate actual and suspected functions of the RES.

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FIGURE 2. Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000

FIGURE 3 Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000

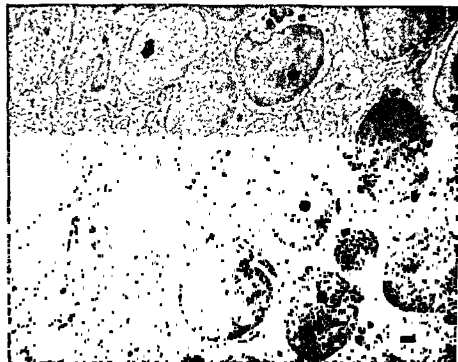


FIGURE 1 Micron bar (right) denotes approximate magnification, 1 mm. is equivalent to ×1000

with alcohol, embedding in methacrylate, polymerizing with a catalyst and heat, sectioning with a Porter-Blum microtome,* and examining the sections in an RCA EMU-2 electron microscope †

FIGURE 2 illustrates, we believe, a reticulum cell in transition to a Sternberg-Reed cell A prominent large nucleus, nucleolus, many small normal-appearing mitochondria, and a few scattered fat granules are present. For size comparison one may note a portion of a lymphocyte at the lower center FIGURE 3 is an electron micrograph of a Sternberg-Reed cell. This cell is much larger than the previous one, its nucleus is irregular, and the cytoplasm contains numerous mitochondria and other structures FIGURE 4 is still [illegible] Sternberg-Reed cell but it shows a degree of mitochondrial degeneration

sitional form of the reticulum cell were presented to the Sixth International Congress of Hematology in Boston, Mass, in 1957, and are being published soon in the proceedings[10] of the Congress so that it is not now necessary to review this in any greater detail FIGURE 5 illustrates another Sternberg-Reed cell with a distorted and irregular nuclear membrane and two bizarre

* Product of Ivan Sorvall, Inc, Norwalk, Conn

† Product of The Radio Corporation of America, Camden, N J

FIGURE 2 Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000

FIGURE 3 Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000

FIGURE 4 Micron bar (right) denotes approximate magnification; 1 mm is equivalent to ×1000

FIGURE 5 Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000

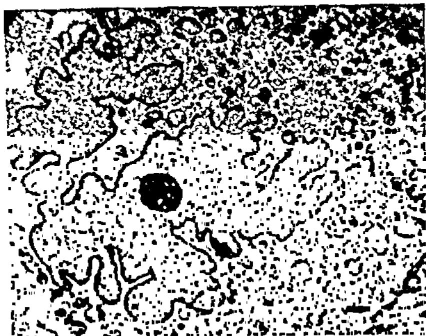


FIGURE 6 Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000

nucleoli Two large lipid granules, as well as degenerate mitochondria and other structures, are present in the cytoplasm FIGURE 6 is a portion of another Sternberg-Reed cell having a much more aberrant nucleus than the previous cells It is bilobate and has a very irregular nuclear membrane, a prominent nucleolus, and scattered nonhomogeneous chromatin

The Sternberg-Reed cell of FIGURE 7 has the usual large, irregularly indented nucleus, prominent nucleoli, mitochondria, cytoplasmic fat granules, and some other nuclear structures that one might be tempted to call nuclear inclusion bodies or viruslike inclusions At higher magnification (FIGURE 8) the nuclear membrane, the cytoplasm, and the mitochondria are easily recognized The previously mentioned nuclear structures now appear to consist of a membrane enclosing some particles By means of serial sectioning these structures can be identified as mere extensions or projections of the cytoplasm into the nucleus

In serial sectioning, each section is cut from the block of tissue so that the corresponding areas of several subsequent sections can be examined With the ultrathin sectioning microtome one is able to cut sections about 0 05 μ in thickness Thus, if every third section were examined, the cell structure at depth intervals of 0 1 μ could be determined Serial sectioning is illustrated by FIGURES 7 and 9 to 11 FIGURE 7 illustrates a Sternberg-Reed cell in the

FIGURE 7 Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000

FIGURE 8 Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000

FIGURE 9 Micron bar (right) denotes approximate magnification; 1 mm is equivalent to ×1000

FIGURE 10 Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000

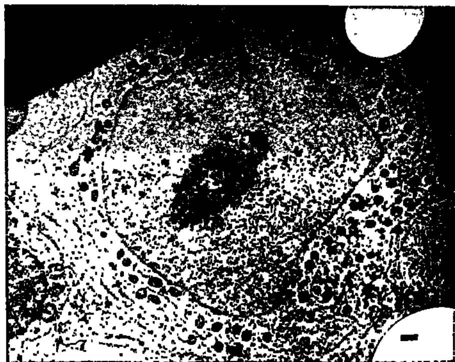


FIGURE 11 Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000

first or uppermost section cut from the block of tissue FIGURE 9 represents the same cell after 10 sections, or about 0 5 μ deep into the cell. FIGURE 10 represents the cell another 0 5 μ deeper The bottom section of the cell, which is another 0 5 μ deeper, is seen in FIGURE 11 The nuclear contour and nucleolar configuration have changed, and some of the cytoplasmic projections or extensions into the nucleus have disappeared

Inasmuch as M J Kopac describes nucleolar reactions elsewhere in these pages, it may be of interest to look at the nucleolus of this same cell at higher magnification FIGURES 12 to 14 illustrate the nuclei in higher magnification in the same serial sequence The cytoplasmic extension or projection near the nucleolus is very evident The nucleolar material is not bounded by a membrane and appears to consist of a disoriented nonhomogeneous material A lipid granule and a mitochondrion are now easily apparent in the cytoplasmic projection in FIGURE 12 The nucleolus appears vacuolated. In some instances the nucleolar material is adjacent to the cytoplasm with only the nuclear membrane separating them In FIGURE 5 both nucleoli appear to border on cytoplasm.

The cytoplasmic components of the Sternberg-Reed cell differ greatly from those of a lymphocyte Normally, a lymphocyte has a regular nuclear membrane, a few small round mitochondria with internal structure, and only a

FIGURE 12. Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000

FIGURE 13 Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000

FIGURE 14. Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000

FIGURE 15 Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000



FIGURE 16 Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000 See text for symbols

small area of cytoplasm (FIGURE 1) FIGURE 15 shows a portion of a Sternberg-Reed cell having an irregular nuclear membrane, many small round mitochondria, one rod-shaped mitochondrion, a few lipid granules, and a large area of cytoplasm In contrast to this cell the cytoplasm of the Sternberg-Reed cell shown in FIGURE 16 is vastly different The cytoplasm appears to consist of numerous vacuoles (V), a rare normal-appearing mitochondrion (M), many degenerate mitochondria and, in the center of the field, a structure (S) consisting of a membrane enclosing several other structures FIGURES 17 to 20 represent this same cell at successive depths of about 0 05 μ If one examines the large round cytoplasmic structure in the serial sections it is apparent that this structure contains some small particles as well as a large one, and that it is like a cylinder in the cytoplasm of the cell If one follows the vacuole (V) through the series it becomes apparent that something is in the vacuole (FIGURE 17) As one sections deeper into the cell this material almost fills the vacuole (FIGURES 18 and 19) and, finally, the material appears to be a mitochondrion (FIGURE 20) Observe the changes in the mitochondrion (M) The mitochondrial cristae are only partially present (FIGURE 16) They became less apparent and, in the bottom section of the sequence (FIGURE 20), it is apparent that the mitochondrion was lying tangentially to the plane of sectioning, and that only two cristae are present

FIGURE 14. Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000

FIGURE 15 Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000

FIGURE 19 Micron bar (right) denotes approximate magnification, 1 mm. is equivalent to ×1000 See text for symbols

FIGURE 20 Micron bar (right) denotes approximate magnification, 1 mm. is equivalent to ×1000 See text for symbols

Figure 17 Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000 See text for symbols

Figure 18 Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000 See text for symbols

FIGURE 22 Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000

FIGURE 23 Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000



FIGURE 21 Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000

FIGURES 21 to 23 (a round reticulum cell, a plasma cell, and a bundle of collagen fibers, respectively) illustrate other structures often present in lymph nodes of patients with Hodgkin's disease. The plasma cell is recognized by its round, regular nuclei, small, dark normal mitochondria, cytoplasmic vacuoles, and structures known as endoplasmic reticula. Adjacent to these are noted the so-called ribonucleic acid (RNA) granules FIGURE 23 shows a bundle of collagen fibers, a portion of which were cross-sectioned and another portion sectioned tangentially

One of the aims in this electron microscopy study of the lymph nodes of patients with Hodgkin's disease was to discover, if possible, whether the Sternberg-Reed cell contained a component or components which, by the physical standards of size, shape, and osmium-tetroxide-staining properties, resembled known viruses Electron microscopists often point out structures present in tissue sections and identify them as viruses or as viruslike structures on the basis of these physical characteristics However, such identifications are not generally accepted as valid, even if they were, in this study no components of the Sternberg-Reed cell were observed which, in our opinion, resembled viral structures It is possible, first, that the virus of Hodgkin's disease, should such an entity exist, may have physical properties different from those expected, second, that it may not be present in the Stern-

FIGURE 22 Micron bar (right) denotes approximate magnification, 1 mm. is equivalent to ×1000

FIGURE 23 Micron bar (right) denotes approximate magnification, 1 mm is equivalent to ×1000

berg-Reed cell, and, finally, it may be that the techniques used were not capable of revealing the virus.

The micrographs presented here indicate that the nucleus of the Sternberg-Reed cell, as well as the nucleolus, is interacting with some cytoplasmic component The many degenerate mitochondria present are associated with the extensive vacuolization noted in the cell. These changes may be interpreted as reflecting some abnormal metabolic activity.

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gation on sections from lymph nodes and bone marrow in malignant blood diseases Blood **12:** 278–294

MALIGNANT NUCLEOLI: CYTOLOGICAL STUDIES AND PERSPECTIVES*

By M. J. Kopac and Gladys M. Mateyko

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Introduction

Atypical nucleoli have been recognized in neoplastic cells by many cytologists. Pianese[1] was probably the first investigator to note an increased nucleolar size in malignant cells. MacCarty[2] also concluded that nucleoli are larger in malignant cells, and MacCarty, Haumeder, and Berkson[3] estimated the ratio of nucleolar area to nuclear area to range from 1:17 to 1:5 in malignant cells and from 1:45 to 1:13 in normal cells. Additional information along similar lines was published by MacCarty[4, 5] and by MacCarty and Haumeder.[6] It should be pointed out that large nucleoli are frequently seen in neurocytes and oocytes, in hepatic cells, or in cells undergoing some hyperactive function, especially secretion.

The more recent studies are those by Long and Taylor[7] on human ovarian

substance, frequently eosinophilic and presumably elaborated by the nucleolus, was pumped from the nucleus to the cytoplasm

were undertaken in order that the possible role of the nucleolus in the neoplastic processes can be more thoroughly evaluated.

It soon became evident that size or number represented only two of the many variations found in the nucleoli of malignant cells. In fact, these two characteristics may turn out to be the least important, since many other and more striking nucleolar manifestations have been discovered. The evaluation of the role of the nucleolus in malignancy requires a consideration of all atypical features and all types of nucleolar lesions. In addition, consideration must be given to the fine structures of the nucleolus, including the nucleolonema, the *pars amorpha*,† and the nucleolar organizers in relation to normal and atypical nucleoli. Most important is the nucleolar-chromosomal complex, which may turn out to be one of the fundamental regulatory mechanisms in the cell.

* The investigations reported in this paper were supported in part by Grants C-2018 and C-3490 from the National Cancer Institute, Public Health Service, Bethesda, Md., and Grant DRG 271 from the Damon Runyon Memorial Fund for Cancer Research, Inc., New York, N. Y.

† See below, under Nucleolar Structures.

berg-Reed cell; and, finally, it may be that the techniques used were not capable of revealing the virus

The micrographs presented here indicate that the nucleus of the Sternberg-Reed cell, as well as the nucleolus, is interacting with some cytoplasmic component The many degenerate mitochondria present are associated with the extensive vacuolization noted in the cell. These changes may be interpreted as reflecting some abnormal metabolic activity.

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tumor cells from the frog adenocarcinoma were useful for the transplantation of nucleoli

nucleolus and its chromosome may, under certain conditions, lead to an unregulated production of both nucleolar material and chromatin.

The various observed nucleolar states were integrated into a scheme that

be no more meaningful than were isolated studies of cells in karyokinesis before the mitotic cycle was established. By now, however, the chromosomal cycle is fairly well established. Unfortunately, a similar consideration concerning nucleoli is not yet available, although the scheme presented here is an attempt to provide this.

Finally, we include some notes on the transplantation of nucleoli from one cell to another, together with the methods devised for studying immediate or short-term changes following nucleolar transplantation. The main purpose of this report is to provide a morphologic background for future studies on nucleoli that may elucidate the possible function of these structures in both normal and neoplastic cells.

Nucleolar Structures

Although the nucleolus in the interphasic nucleus seems to be a reasonably simple structure, there is as yet no universally accepted interpretation of either its structure or function. The earlier studies of nucleolar morphology have been reviewed by Montgomery,[10] by Gates[11] and, more recently, by Vincent[12] and by Hertl.[13] Many of the earlier studies have recognized the presence of vacuoles within nucleoli, while others refer to the presence of nucleolini. The former structures are chromophobic, while the latter stain intensely with several dyes. The evolution of nucleolar vacuoles in human hepatic cells was studied by Macary.[14] The vacuolar material generated by the nucleolus is extruded through the nuclear membrane with the formation of a clear perinuclear ring in the cytoplasm. Cytochemical studies of these vacuoles were negative for lipases, acid or alkaline phosphatases, nucleic acid bases, purine bases, nucleic acid phosphate, proteins, amino acids, and iron. The vacuoles sometimes contained fine fatty granulations. Chromophobic vacuoles are frequently seen in neoplastic cells, in some instances, vacuoles of the same size and position become intensely stained by eosin. Several examples of nucleolar vacuolation will be illustrated and described later.

One of the most illuminating concepts of nucleolar morphology is that elabo-

Included in this report is a summary of many detailed cytological studies of nucleoli made on the cells from human ovarian and other gynecologic neoplasms, augmented by observations on cells of the adenocarcinoma of the frog kidney, melanotic and amelanotic cells from Xiphophorin fish melanomas, and from cells of lymph nodes obtained from Hodgkin's diseased patients and grown in tissue culture.

Portions of surgically removed human tumors were sent to our laboratory by messenger from the College of Physicians and Surgeons, Columbia University, New York, N Y. through the courtesy of Howard C. Taylor, Jr A preliminary diagnosis of tumor and grade of malignancy was made by members of the staff of the Department of Obstetrics and Gynecology, with final diagnosis confirmed by the Department of Pathology of the College of Physicians and Surgeons We now have nucleolar, micrurgical, and cytochemical information on isolated cells and tissue cultures of over 200 human gynecologic neoplasms. Included are 3 thecomas, 2 of the rare disgerminomas, over 65 serous papillary cystadenocarcinomas, and 55 pseudomucinous tumors (all ovarian)

Most of the specimens of ovarian neoplasm received at our laboratory are grown in tissue culture, either as roller tube (fluid media) or Maximow double cover-glass preparations (fluid and solid media) Special mention is made of the usefulness of sterile human ascites fluid, both homologous and heterologous, as a substitute for serum in the culture media The ascites fluid of ovarian malignant origin and specifically from metastatic serous papillary cystadenocarcinomas not only provides an excellent growth medium for human tissues, but also supports the growth of piscine epidermal cells and amphibian kidney cells, both normal and neoplastic

While 37° C is the optimal temperature for cultures of human tissues, it has been found that well-proliferated cultures can be kept safely at 4 to 6° C for periods of several weeks without renewing the culture medium The storage or "hibernation" period can be terminated at any time by returning the cultures to room temperatures After the cultures have been kept at room temperature for 24 hours they can be incubated at 37° C, the medium renewed, and proliferation once more ensues This simple procedure extends the period during which cultures of special material can be made available, especially for micrurgical or cytochemical studies

The material studied included conventional histological sections of the neoplasms, smears taken from solid tumors or from separated cells, and also those recovered from ascites fluids and tissue cultures The cells, following the use of appropriate fixatives, were stained with hematoxylin and eosin or phloxine, methyl green and pyronin Y, Schleifstein's technique (basic fuchsin and methylene blue), eosin and methylene blue, Feulgen reaction, aceto-orcein and fast green FCF, gold and silver impregnation, and supravital staining with methylene violet (Bernthsen) and other thiazine dyes When fixed in cold methanol and stained with methyl green and pyronin Y (Long and Taylor[7]) smears of tumor material are most useful for the study of nucleoli and chromatin structures Samples of solid tumors subjected to mild mechanical agitation, trypsin digestion, or both, provide suspensions of separated cells (Kopac[8])

tumor cells from the frog adenocarcinoma were useful for the transplantation of nucleoli.

Some of the nucleolar atypia seen in malignant cells also may be found in the non-neoplastic cells. However, these atypia are more intense and occur more frequently in malignant cells. There is perhaps one feature that ultimately may distinguish the nucleoli of normal and malignant cells. This feature involves the nucleolar-chromosomal complex in which dissociation between the nucleolus and its chromosome may, under certain conditions, lead to an unregulated production of both nucleolar material and chromatin.

The various observed nucleolar atypia were integrated into a scheme that serves as a working hypothesis for the many morphologic and, possibly, physiologic manifestations of nucleoli in both normal and malignant cells. Without such a scheme, even though provisional, the various nucleolar aberrations would be no more meaningful than were isolated studies of cells in karyokinesis before the mitotic cycle was established. By now, however, the chromosomal cycle is fairly well established. Unfortunately, a similar consideration concerning nucleoli is not yet available, although the scheme presented here is an attempt to provide this.

Finally, we include some notes on the transplantation of nucleoli from one cell to another, together with the methods devised for studying immediate or short-term changes following nucleolar transplantation. The main purpose of this report is to provide a morphologic background for future studies on nucleoli that may elucidate the possible function of these structures in both normal and neoplastic cells.

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One of the most illuminating concepts of nucleolar morphology is that elabo-

rated by Estable and Sotelo[15, 16] These investigators postulated that the nucleolus consists of two main components; that is, a filamentous structure called the nucleolonema, and the other more bulky but homogeneous structure called the *pars amorpha*

According to these authors, the nucleolonema can be made visible by gold or silver impregnation, by ferrocyanide techniques, by postvital staining with pyronin and, in living cells, by phase-contrast (Denues and Mottram[17]), dark-field, or transmitted illumination. Furthermore, a filamentous, reticulated, or laminated structure within the nucleolus has been demonstrated by electron microscopy (Borysko and Bang,[18] Bernhard *et al*,[19] Rozsa and Wyckoff,[20] Porter[21] and, more recently, by Novikoff[22])

[illegible]

Estable and Sotelo[16] stated that a nucleolus may contain several nucleolonemal tufts in contact with each other by bridges. Several independent nucleolonemata are formed if these bridges are broken Other variations in size and shape of the nucleolonema may occur during mitosis, cell growth and differentiation, different degrees of cellular activity, and in the course of pathologic processes According to these authors these changes are morphologic, chemical, and physicochemical in nature

The structural changes in the nucleolonema are the most striking during mitosis From the tightly woven tuft as the nucleolonema exists during interphase, an unfolding occurs during the prophase The nucleolonema becomes thinner and spreads throughout the nucleoplasm and comes close to the chromosomes. It grows during the premetaphase and metaphase and becomes associated longitudinally with the chromatids When the chromosomes separate during anaphase each one has its corresponding nucleolonemal segment During telophase the nucleolonema thickens and becomes shortened and finally assumes the compact glomerular configuration characteristic of interphasic nucleoli. The *pars amorpha* disappears during mitosis and reappears during the telophase as a condensation of substance that accumulates around the glomerular tuft

The law of origin of the nucleolonema, according to Estable and Sotelo, is *omnis nucleolonema e nucleolonema* * Furthermore, the lack of a chromosome does not interfere with the existence, growth, or proliferation of the nucleolonema. Consequently, this structure is to be considered as one of the five fundamental structures common to all germinal or somatic cells

Sosa[23] has studied the argentophilic spherules frequently seen within the nucleoli of large neurons (pyramidal, Purkinje, mitral, and anterior-horn multipolar cells) of birds and mammals These structures are Feulgen negative and should not, therefore, be considered as "elementary chromosomes" The spherules may become connected into a network of variable complexity and extension It is probable that the structures described by Sosa are similar to the nucleolonema if not identical with it

* Every nucleolonema derives from [another] nucleolonema

THE NUCLEOLAR-CHROMOSOMAL COMPLEX

The relationship of nucleoli to certain chromosomes by way of the nucleolar organizer has been extensively studied by McClintock,[24] by Kaufmann,[25] and by Matsuura,[26] and has been reviewed by Kaufmann[27]

Nucleolar organizers in the mouse cells have been studied recently by Ohno *et al*[28] In the mouse, only the X and Y chromosomes are found in association with the nucleolus, the nucleolar organizer being located near the kinetochores Ohno and Kinosita[29] found that the nucleoli in rat lymphoblasts are organized by a few autosomes In mesothelial cells of the rat omentum, Ohno and Kinosita[30] showed an attachment of certain chromosomes at their subterminal regions to the nucleolus, thus resembling nucleolar chromosomes with SAT*-like zones described in other organisms

Schultz and St. Lawrence[31] have produced a cytological map of the autosomal nucleolar chromosome in man following a study of the pachytene male chromosomes during spermatogenesis In addition, Schultz and St. Lawrence reported that the X chromosome also forms a nucleolus Thus, in the diploid cells of the human female one might find as many as four nucleoli organized from four separate nucleolar chromosomes On the other hand, following fusion of one or more nucleoli, it would be possible to find one, two, or three nucleoli Reversible fragmentation and fusion of nucleoli during the telophase have been reported in *Trillium* by Matsuura[26] and in *Tmesipteris* by Yeates.[32]

There is no need, at this time, to define the precise nature of the nucleolar organizer nor to limit the number of genes, if any, to the function of forming a new nucleolus during telophase Schultz and St Lawrence have shown that the nucleolar organizer in the human autosome is definitely associated with the tenth and eleventh chromomeres of a chromosome that has twenty-two recognizable chromomeres This position seems to be fairly constant and, furthermore, the chromomeres to which the nucleolus is attached are unusually large and become densely stained with orcein

The relationship of the nucleolus to the chromosome and to its "cycle" during mitosis would be more intelligible if the nucleolonema (Estable and Sotelo[16]) were continuous with the nucleolar organizer or were part of it Thus, the nucleolenema, the nucleolar organizer, and the nucleolar chromosomes would be the self-replicating structures During mitosis the *pars amorpha* disappears, as postulated by Estable and Sotelo but, during mitosis, the nucleolonema, the nucleolar organizer, and its chromosome must be replicated During reconstitution of the nucleus, following the early telophase, the nucleolonema becomes condensed and entangled to form a tuft that then presents a substrate on which the new *pars amorpha* accumulates If the *nucleolar-chromosomal complex is correctly interpreted, then it could be that* this complex not only determines the number of nucleoli, but the number of nucleolar chromsomes as well In addition, the onset of replication must be mutually controlled in that one cannot replicate without the other

The significance of the nucleolar-chromosomal complex in the neoplastic process will be discussed below

* SAT stands for "sine acido thymonucleinico" An SAT chromosome is one that is associated with the formation of the nucleolus or that possesses one or more satellites

Nucleolar Lesions in Neoplastic Cells

Human ovarian tumor cells. The term nucleolar lesion is used to designate any atypical manifestation of the nucleolus, either morphologic, cytochemical, or functional. Various nucleolar structures and behaviors were noted in the study of cells of serous papillary cystadenocarcinomas of the human ovary, and these variations are summarized in FIGURE 1. Some of these nucleolar features can be seen with some modification in normal cells, others may appear in cells undergoing some active phase such as secretion or protein synthesis, and still others have been seen regularly only in neoplastic cells The more striking nucleolar manifestations are summarized in FIGURES 1 and 2, while other examples are shown in FIGURES 3 to 8

In the human ovarian cystadenocarcinoma cells, the nucleoli make their first appearance during telophase In FIGURE 1*a*, the new nucleoli are initially visible as small, solid, strongly pyroninophilic structures One incipient daughter cell, shown here, has three small nucleoli, while the other has only two Following the regrouping of telophase chromosomes and before the reconstitution of a nuclear membrane, the nucleoli become larger, and some fusion may occur. For example, in FIGURE 1*b* one incipient daughter nucleus has a single large nucleolus with vacuoles, while the other incipient nucleus shows three smaller solid nucleoli

During the interphase, the nucleolus may undergo a variety of changes It may become hypertrophied without vacuolization, as in FIGURE 1*c* In others, the nucleolus may hypertrophy with considerable vacuolation, as in FIGURE 1*d* Following extensive vacuolation, both internal and marginal, the hypertrophied nucleolus may assume bizarre shapes, as in FIGURE 1*e*. Frequently the marginal vacuoles will rupture to produce still more bizarre shapes Highly vacuolated nucleoli, as in FIGURE 1*f*, may separate into irregular masses that may or may not be joined by beaded strands of pyroninophilic material Some nucleoli may also show the formation of buds or satellites.

The large nuclei will frequently become multinucleolate, as in FIGURE 1*g* This nucleus has twenty-one nucleoli of various sizes, ranging from the small ones seen in telophase to the large ones seen in interphase Such multinucleolate situations may arise by fragmentation of large nucleoli (FIGURE 1*f*) or through the presence of more than the usual number of nucleolar organizers and chromosomes According to the studies of Schultz and St. Lawrence,[31] cells of the human female should have a maximum of four nucleolar organizers,

suggesting that the formation of vacuoles is perhaps an independent function

distribution of nucleoli can best

cause of a presumably common



ancestry and identical cytoplasmic environment, should have the same nucleolar pattern Barring any mitotic incidents that might lead to monosomic or trisomic nucleolar chromosomes, both nuclei should have the same chromosomal complement and hence the same nucleolar configuration As reported by

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material

The large nuclei will frequently become multinucleolate, as in FIGURE 1*g* This nucleus has twenty-one nucleoli of various sizes, ranging from the small ones seen in telophase to the large ones seen in interphase Such multinucleolate situations may arise by fragmentation of large nucleoli (FIGURE 1*f*) or through the presence of more than the usual number of nucleolar organizers and chromosomes According to the studies of Schultz and St. Lawrence,[31] cells of the human female should have a maximum of four nucleolar organizers, that is, one on each of two autosomal chromosomes and on two X chromosomes In FIGURE 1*g*, even if the smaller nucleolar masses were derived by budding or fragmentation, there still remain more than four reasonably typical nucleoli Presumably, in this nucleus there must have been more than the euploid number of nucleolar chromosomes The extraordinarily large size of the nucleus would suggest a polyploid condition In this nucleus, two nucleoli show the formation of small satellites by budding Only a few of the nucleoli are vacuolated, suggesting that the formation of vacuoles is perhaps an independent function of each nucleolus

The variability in size, number, shape, and distribution of nucleoli can best be compared in binucleated cells which, because of a presumably common

seen frequently in the nucleoli of neoplastic cells (FIGURE 1*d*, *e*, and *f*) It might be that these vacuolar inclusions become strongly eosinophilic under certain conditions and then appear as pink to bright red inclusions in cells stained with hematoxylin and eosin or phloxine Older references in the literature mention the nucleolinus as a small intranucleolar inclusion that stains intensely with various dyes It is unlikely, however, that the eosinophilic inclusions observed in the tumor cells are the same as the nucleolinus.

There is no striking constancy in the number of eosinophilic inclusions found in either the nucleoli, the nuclei, or the cytoplasm As many as five inclusions have been seen in the nucleolus There may be five or six larger eosinophilic bodies in the nucleus Frequently, several inclusions may be seen in both the nucleolus and in the nucleus that suggest that the eosinophilic material is repeatedly elaborated by the nucleolus In the cytoplasm one usually finds a single large mass, although in binucleated cells there may be two masses of approximately the same size and staining density

Several stages in the development of eosinophilic bodies were seen in cells grown in tissue culture from a serous papillary adenocarcinoma (grade III) of the human ovary First is the appearance of bright eosinophilic bodies in a strongly basophilic nucleolar body. The substance is then extruded by the nucleolus, and both are surrounded by a common chromophobic corona. Later, [illegible] ompact at first, [illegible] Frequently, a [illegible] leolus, which is always vacuolated

The smaller bodies may appear as doublets in the nucleus, giving the impression that they may pinch in two The bodies vary in shape from spheres to rods, when large, they become irregular, almost amoeboid, in shape These nuclear inclusions become larger and are eventually extruded from the nucleus into the cytoplasm (see FIGURE 2*a*) The eosinophilic masses in the cytoplasm are large, and their staining capacity may vary from faint pink to bright red. *These masses are also surrounded by a chromophobic corona, no matter whether* they are in the nucleus or in the cytoplasm When in the cytoplasm, the eosinophilic extrusions may become vacuolated, and may even persist in the cytoplasm during mitosis. In one cell, a large mass was seen on the polar side of one set of anaphase chromosomes with its chromophobic corona extended to the chromosomes.

In tissue cultures of another tumor (papillary serous adenocarcinoma, cells from paracentesis fluid, grade III), the intranuclear inclusions were vacuolated The shapes of these bodies also varied from spherical bodies, as they appear inside the nucleolus, to rods or highly irregular shapes In this tumor many nucleoli contained small, compact, and spherical eosinophilic inclusions In some nuclei, rod-shaped bodies seemed to be spun out by the nucleolus Occasionally there were suggestions that these intranuclear inclusion bodies may also divide by pinching in two

In binucleated cells, where the eosinophilic inclusions appear within the nucleoli of both nuclei, their development in the two nuclei may be almost synchronous The bodies are surrounded by extensive chromophobic coronas,

Kopac and Mateyko,[33] approximately 54 per cent of binucleated cells from a serous papillary cystadenocarcinoma, grown in tissue culture, show similar nucleolar number (1 to 8), size, shape, and position, while 46 per cent of binucleated cells show unmistakable dissimilarities.

Mechanisms of nuclear anisogenicity may be related, among others, to mitotic incidents such as tripolar, tetrapolar, and even pentapolar spindles. In addition, the lagging chromosomes, if involved in nucleolar organization, would thus account for nucleolar anisogenicity. The fusion or fragmentation of reconstituting nucleoli also may be partly a genic or nucleolar organizer response. The size, shape, and position of nucleoli in these binucleated cells are more variable than number, since 72 per cent of such cells will have the same number of nucleoli in each nucleus.

Similar studies were made on binucleated cells occasionally found in smears from tumors or in paracentesis fluids by Jaffe,[34] who found that the three most variable parameters in reference to nucleolar variability are number, size, and distribution. The least variable parameter is shape, since nucleoli probably tend toward a spheroidal configuration. For example, where 73 per cent of the binucleated cells may have nucleoli of different sizes and 83 per cent may have different distributional patterns of such nucleoli, 87 per cent of the cells will have nucleoli of essentially the same shape. In FIGURE 1*h* both nuclei have essentially the same nucleolar pattern in reference to number, size and, to a certain extent, distribution and shape. On the other hand, in FIGURE 1*i*, the two nuclei have different numbers of nucleoli, one having eight nucleolar masses while the other one has but four. In both examples there is vacuolation within some of the nucleoli in each nucleus.

Interphase nucleoli, either with or without associated chromatin, may form filaments, as shown by FIGURE 1*j*. Fantastic nucleolar configurations are frequently seen in association with large irregular masses of chromatin enclosing spherical aggregates of alveolar material that is faintly pyroninophilic; such a bizarre nucleolar configuration is shown in FIGURE 1*k*. The association of amorphous lumps of chromatin with alveolar masses is common, as are chromatin rims. Ludford[15] has also studied nucleolar associated masses of chromatin in tissue cultures of tumor cells (see also Hertl[13]). In addition, it is not uncommon to find large nucleolar masses, either solid or vacuolated, in close proximity with amorphous chromatin and alveolar masses. The alveolar material apparently is extruded by the nucleus into the cytoplasm. Numerous instances of alveolar and pyroninophilic inclusions have been seen in the cytoplasm and, in one instance, the actual process of extrusion was visible.

Nucleoli, *in toto*, may be extruded into the cytoplasm, as in FIGURE 1*l*, otherwise the filamentous structures may be extruded, as in FIGURE 1*m*. In the latter figure the nucleolar residue inside the nucleus is associated with amorphous chromatin. The extrusion of chromatin along with the nucleolar material, however, has not been seen.

In addition to the nucleolar atypia already described there is another aberration seen in serous papillary cystadenocarcinomas of the human ovary. Bright eosinophilic inclusions can be found in some of the nucleoli. These inclusions are of the same size and position as the chromophobic vacuoles, as may be

and at first they occupy such zones jointly with the nucleoli. Migration of these bodies away from the nucleolus occurs later, and there is some increase in size of the bodies as they migrate toward the nuclear periphery in both nuclei The development and subsequent extrusion of eosinophilic bodies into the cytoplasm is almost synchronous.

In another binucleated cell there were several eosinophilic bodies in the larger, extensively vacuolated nucleoli. In addition, one nucleus had a compact intranuclear inclusion, and the extrusion of another irregular or amoeboid mass into the cytoplasm was also evident. The other nucleus had a fairly large cytoplasmic mass adjacent to it. In another binucleated cell there were two eosinophilic masses, each of which was adjacent to a nucleus and in about the same relative positions in the cell. These observations suggest still further the possibility of synchrony in the two nuclei

The significance of these eosinophilic inclusions is unknown; however, there [illegible]

cytoplasm Although the eosinophilic material, when in the nucleus or cytoplasm, is surrounded by a chromophobic corona, the early intranucleolar inclusion bodies are not surrounded by a clear zone One or several compact, strongly eosinophilic bodies are visible embedded in the basophilic carcass of the nucleolus Whatever their significance, these eosinophilic bodies may be the manifestation of a nucleolar and nuclear pump, as reported by Duryee[8] in connection with the Lucké tumor cells.

Cells of Lucké Adenocarcinoma. The nucleoli of cells from the renal adenocarcinoma of the frog, grown in tissue culture, also show considerable variability in size, shape, and activity FIGURE 2*a* shows several eosinophilic masses in the cytoplasm of a multinucleated cell Also shown is one mass being extruded into the cytoplasm by the nucleus, with a fairly large portion still remaining inside the nucleus This eosinophilic material, whether nuclear or cytoplasmic, is always surrounded by a chromophobic corona The inclusions seem to originate in the nucleolus and apparently appear as compact eosinophilic granular inclusions in otherwise vacuolated nucleoli

Pleomorphic nucleoli, frequently intensely vacuolated, are shown in FIGURE 2*b* to *q* Some of these are shown surrounded by extensive chromophobic coronas, as in FIGURE 2*g*, *h*, *l*, *m*, *o*, *p*, and *q* All nucleoli, however, are surrounded by chromophobic coronas, and some, as in FIGURE 2*q*, are extensive The nucleolus in FIGURE 2*q* appears to have pulled apart to form two masses connected by persisting strands It is amazing how extensive vacuolation may be For example, the nucleoli shown in FIGURE 2*b*, *e*, and *k* are not much more than a cluster of vacuoles with only the thin vacuolar walls persisting to maintain the structure

Aceto-orcein and fast green FCF stains highlight the nucleolar-associated chromatin when present in Lucké tumor cells The nucleoli may be rimmed by a shell that stains intensely with orcein (chromatin) Even the outer contours of a pleomorphic nucleolus may be completely surrounded by a shell of chroma-

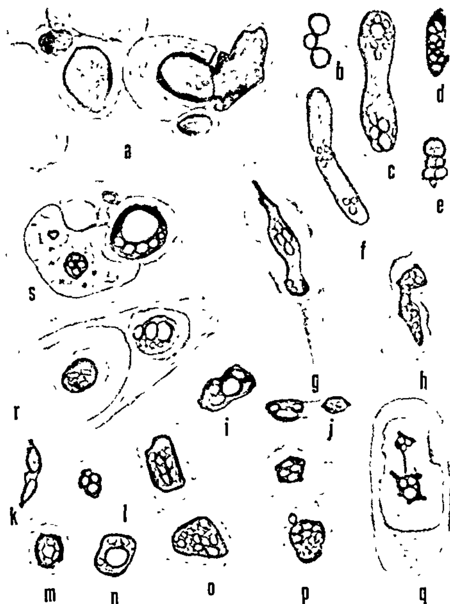


FIGURE 2 Nucleolar lesions in renal adenocarcinoma cells of the frog Original drawings in water color, taken from tissue cultures stained with hematoxylin and eosin Descriptions of structures are included in the text ×1150

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instances of extrusion from nucleolus to nucleus, as well as from nucleus to cytoplasm Although the eosinophilic material, when in the nucleus or cytoplasm, is surrounded by a chromophobic corona, the early intranucleolar inclusion bodies are not surrounded by a clear zone One or several compact, strongly eosinophilic bodies are visible embedded in the basophilic carcass of the nucleolus Whatever their significance, these eosinophilic bodies may be the manifestation of a nucleolar and nuclear pump, as reported by Duryee[8] in connection with the Lucké tumor cells

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tin. The nucleolar masses are uniformly stained by the fast green FCF. Other nucleoli are not rimmed by chromatin; instead, small masses of it are associated with the nucleolus, and as many as five to seven of such masses may be found. Other nucleoli are rimmed by chromatin and, in addition, a number of smaller spheroidal chromatin masses are attached to the rim It is not possible to tell whether these smaller masses are chromosomes, but they might be. Hertl[13] has described paranucleolar Feulgen-positive masses in hypothalamus cells, but these are somewhat different in form and behavior from those seen in Lucké tumor or human ovarian cancer cells The nucleolar-associated chromatin in rat lymphocytes apparently represents the autosomes responsible for organizing the nucleolus (Ohno and Kinosita[26]).

Cells stained with hematoxylin and eosin will also show masses or rims associated with the nucleoli that are generally more acidophilic than basophilic; some of the larger nucleoli may have as many as ten small spheroidal basophilic masses attached to them These particles are strongly basophilic and cannot be distinguished from the regular chromatin masses distributed elsewhere in the nuclei

Extruded nucleoli are also found in Lucké tumor cells, as in FIGURE 2*r* and *s* These two extruded nucleoli are vacuolated and surrounded by chromophobic coronas in the cytoplasm. In FIGURE 2*r* the extruded nucleolus and the nucleolus, *in situ*, have approximately the same color as when stained with hematoxylin and eosin. On the other hand, in FIGURE 2*s* the extruded nucleolus is much less basophilic than nucleoli *in situ*

The larger nucleolus, shown *in situ* in FIGURE 2*s*, is surrounded by a shell of small vacuoles with basophilic walls, and the substance contained by these vacuoles may be responsible for the chromophobic corona that frequently surrounds the nucleoli, especially those that show some type of lesion In other words, this nucleolus and its associated structures may be the intermediate stage that leads to the development of the perinucleolar chromophobic coronas

The intranucleolar vacuoles are generally chromophobic, however, these bodies may occasionally show a rather pronounced eosinophilia similar to those seen in the nucleoli of human ovarian cystadenocarcinoma cells Eosinophilic inclusions are made more evident by staining the nucleoli with eosin and methylene blue The small inclusions are red, while the main bulk of the nucleolus is blue, indicating its basophilic nature

The extrusion of material from the nucleus to the cytoplasm is fairly common, and it has been described and illustrated by Duryee.[8] We have also seen numerous examples of this event Many cells can be found with large eosinophilic masses in the cytoplasm, always surrounded by extensive chromophobic coronas The density of staining with eosin varies from cell to cell, ranging from bright pink to orange to faint pink The large eosinophilic masses are frequently vacuolated It is possible that differences in texture and staining density, as well as the presence of vacuoles, merely represent stages in the disintegration or utilization of the inclusion substance, whatever its composition may be

Formation of Filamentous Structures by Nucleoli

Several examples of filaments formed from nucleoli of serous papillary cystadenocarcinoma (grade III) cells of human ovarian cells are shown in FIGURE 3. Either simple or complex filamentous structures may appear as products of the nucleolus. These structures are all pyroninophilic and may or may not be associated with methyl green staining material, presumably chromatin or desoxyribonucleic acid (DNA). The first stages are shown by

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FIGURE 3*d*, *e*, *f*, *h*, *j*, and *k* are more complicated branched filaments, and each one is associated with chromatin. The configuration of the chromatin residue may vary considerably. In some, the residue exists as amorphous masses (FIGURE 3*j*) or as smaller spherules (FIGURE 3*e*), or as alveolar masses (FIGURE 3*h*, *g*, and *i*). The nucleolar filaments may exist as single threads (FIGURE 3*i* and *k*) or as spherules joined together by a thread (FIGURE 3*e*, *h*, *j*, and *l*). FIGURE 3*k* includes a single filament emerging from a small vacuolated nucleolar body rimmed by a shell of chromatin. Associated with this nucleolus is an

The formation of filaments is evident in two instances, and in one of these there is a suggestion of replication of the beaded filamentous structures. FIGURE 3*n* consists of an extraordinarily complicated and tangled filamentous tuft. One might postulate that replication has already occurred several times in this specimen. A somewhat less complex filament is shown by FIGURE 3*p*. Replication is suggested in FIGURE 3*j* and *o*, although here the configuration is complicated. This filament is associated with several masses of amorphous chromatin. All these filaments presumably represent the nucleolonema with relatively small amounts of *pars amorpha*. The significance of the nucleolonema to nucleolar replication has been mentioned previously and is discussed in greater detail below.

On the basis of filament formation, there is the possibility that there may be at least two types of nucleoli. Some of these consist of the nucleolonema and *pars amorpha*, while the others consist only of *pars amorpha*. Since there is a suggestion of replication of the filaments, there is the further possibility that the formation of filamentous structures or thin strands from the nucleoli involves the unraveling of the nucleolus, with the nucleolonema as the guide. It is possible also that the resulting filaments consist mainly of a core represented by the nucleolonema and a thin coating of *pars amorpha*. The formation of filamentous configurations would, therefore, be limited to those nucleoli that contain the nucleolonema. Those nucleoli lacking the nucleolonema could not form filaments, but they might still form vacuoles and become pleomorphic or fragmented.

The nucleoli of mesenchymal chick fibroblasts, in tissue culture, can be induced to form filamentous structures through the action of adenosine

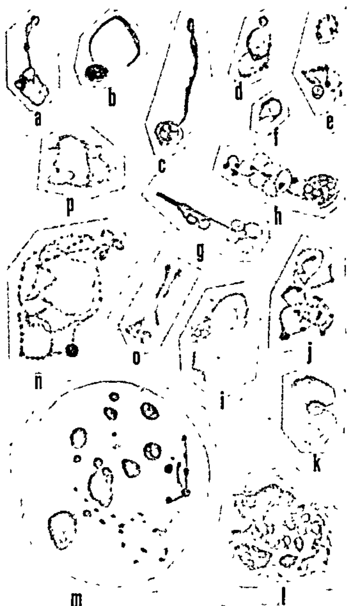


FIGURE 3 Formation of filamentous structures by nucleoli (same tumor as in FIGURE 1) Original drawings in water color, taken from smears stained with methyl green and pyronin Y Description of structures is included in the text ×1500

(Lettré[37]). Even without added adenosine such nucleoli will undergo some transformation from dense, compact nucleoli to the loose, filamentous structures Occasionally, two filamentous structures may be seen in a single nucleus Filamentous structures can also be induced through the action of adenosine on HeLa cells, human myoma, and mouse mammary carcinoma cells Wilson[15] has included the description of several examples of spiremelike configurations emerging from nucleoli, especially in vertebrate oocytes

It should be pointed out that the filamentous structures as described by Lettré[37] are Feulgen positive and are also stainable by methyl green These structures, therefore, are identical neither with the filaments described in human ovarian cystadenocarcinoma cells nor with the nucleolonema of Estable and Sotelo[16] The filamentous structures that emerge from the nucleoli, as shown in FIGURE 3, are pyronin positive and methyl-green negative In reference to the action of adenosine, it would seem that this agent must promote the dispersal of the amorphous ribonucleic acid (RNA)-protein components of nucleoli, in contrast to the action of cobalt ions, which prevent the dispersal of the main nucleolar bulk (Heath[39]) There is a question whether the formation of filaments may not be a partial recapitulation of events that occur normally during mitosis, that is, the disappearance of the *pars amorpha* and the persistence and replication of the nucleolonema. In fact, one might consider that the formation of the filaments from a nucleolus during the interphase may be an attempt to recapitulate reproduction of the nucleolar elements, especially when the nucleolus is separated from the chromsome to which it is normally attached

The formation of branches similar to the simple filaments shown in FIGURE 3*a*, *b*, and *c*, has been studied by Hsu[40] in the pial cells taken from the newborn kitten and grown in tissue culture During prophase an irregularly shaped nucleolus produced a small side branch first appearing as a knoblike projection *near the base of one of the original larger branches, later a second small branch* had developed by the side of the first one Both branches broke away from the nucleolus, and the pieces were seen to wander in the nucleus for some time

During the rotation of the nucleus many small branches were produced and detached from the nucleolus The nucleolus had been constantly changing its shape, becoming smaller, and acquiring a dense periphery and a pale center showing a much lower density (as viewed by phase-contrast optics) The nucleolus disappeared at about the time the nuclear membrane disappeared During early mitosis the nucleolus of the pial cell is highly pleomorphic, constantly changes its shape, and forms branches that break off from it The disappearance of the nucleolus is preceded by a striking decrease in density as viewed by phase contrast

The study of pial cells indicates that pleomorphic and large nucleoli may be found in nonmalignant cells The formation of filamentous structures is probably a property possessed by many types of nucleoli The disappearance of the main [illegible]

[illegible]

an attempt will be made to increase the amount of filament-forming material

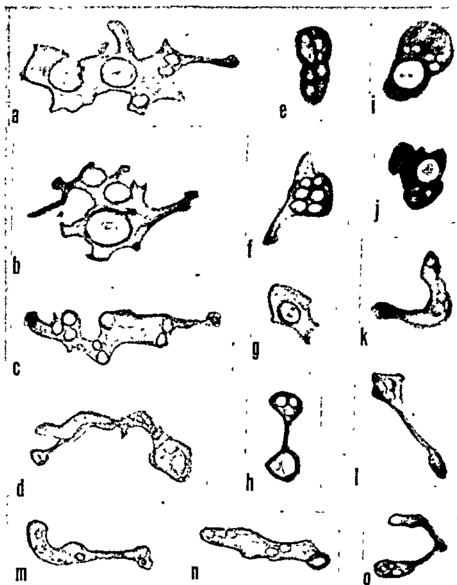


FIGURE 4 Pleomorphic nucleoli (serous papillary cystadenocarcinoma of human ovary, grade III, P & S No 39566) Original drawings in water color, taken from tissue structures stained with hematoxylin and eosin Description of nucleoli is included in the text ×1350

in such cells. The association of filamentous material with amorphous chromatin may be another factor, this is discussed below

PLEOMORPHIC NUCLEOLI

The nucleoli from serous papillary cystadenocarcinomas of the human ovary (grade III) frequently become grossly pleomorphic in tissue culture In

FIGURE 4*a*, *b*, *c*, and *d* the nucleoli are huge and strikingly modified by extensive vacuolation, both internal and marginal. There is a great variation in the size of the vacuoles, the larger ones presumably being derived by the coalescence of the smaller vacuoles. The large marginal vacuoles following the break-through of the vacuolar wall undoubtedly contribute to the bizarre architecture of these nucleoli. The more compact, vacuolated nucleoli are shown by FIGURE 4*e*, *f*, *g*, *i*, and *j*. Elongated nucleoli are shown by FIGURE 4*b*, *m*, and *n*. Elongated nucleoli suggesting fission are illustrated by FIGURE 4*h*, *l*, and *o*.

These nucleoli are frequently eosinophilic, although the vacuolar content is chromophobic. As a rule, the vacuoles within the pleomorphic nucleoli vary in size, some being small, others being large. These inclusions are chromophobic, in no instance have the small eosinophilic inclusions been seen in these nucleoli. On the other hand, without exception the pleomorphic nucleoli are always surrounded by extensive chromophobic coronas.

Less pleomorphic nucleoli are seen in cells of the Lucké adenocarcinoma. On the other hand, the large stromal cells that appear in tissue cultures of Hodgkin's-diseased lymph nodes are as pleomorphic as those seen in cells from ovarian cystadenocarcinomas.

Nucleolar pleomorphism can be induced in mesenchymal chick fibroblasts, HeLa cells, human myoma, and mouse mammary carcinoma cells through the action of adenosine (Lettré[37]). Hughes[41] has observed nucleolar fragmentation in cells of chick tissue cultures through the action of adenosine and moderate fragmentation by benzimidazole or guanosine. Such fragmentation is reversible, and "regeneration" of fragmented nucleoli occurs within 30 to 40 minutes after removal of the nucleoside. Cells with fragmented nucleoli enter prophase and subsequent mitosis. It is quite possible that the factors leading to filament formation or fragmentation are essentially the action of some agent, either intrinsic or extrinsic, on the *pars amorpha* fraction of the nucleolus.

Nucleolar Extrusion

Neoplastic cells. The extrusion of nucleoli has been observed in numerous instances in cells of human ovarian serous papillary cystadenocarcinomas in smears or in tissue culture. Examples of nucleolar extrusion in transit are shown by FIGURE 5*a*, *b*, *c*, and *d*. Large nucleoli may be extruded *in toto*, in some, only the the filamentous portions are extruded, as in FIGURE 5*c* and *d*. Many nucleoli were found in the cytoplasm, and these are essentially similar to the nucleoli *in situ* in regard to compactness or extent of vacuolation.

FIGURE 5*j* shows four nucleolar masses that may have been extruded separately or else a larger nucleolar mass that became fragmented after it was extruded. The former would seem to be the correct interpretation, based on comparison of the sizes of the nucleoli *in situ*, extruded, or in transit as shown in FIGURE 5*b*. In FIGURE 5*l* two almost identical nucleoli are shown in the cytoplasm, possibly indicating a double nucleolar extrusion inside a binucleated cell. It is remarkable that both nucleoli in the cytoplasm have substantially identical vacuoles.

The cytoplasmic or extruded nucleoli are all surrounded by chromophobic

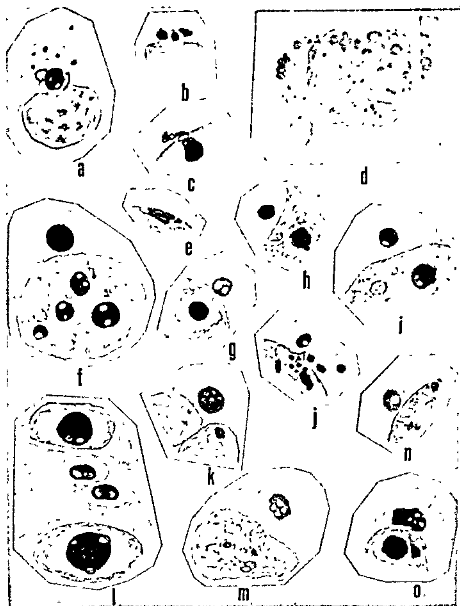


FIGURE 5 Extrusion of nucleoli (same tumor as in FIGURE 1) Original drawings taken from smears stained with methyl green and pyronin Y Nucleoli in cytoplasm as shown here are essentially unchanged Descriptions are in the text ×1000

coronas, such coronas are evident even around small nucleolar fragments In FIGURE 5*d* the obvious extrusion of the nucleolonemal component is also surrounded by a clear and reasonably large corona Nucleolar-associated chromatin was not seen in the extruded nucleoli

Some of the extruded nucleoli may be similar to nucleoli *in situ*, or else there

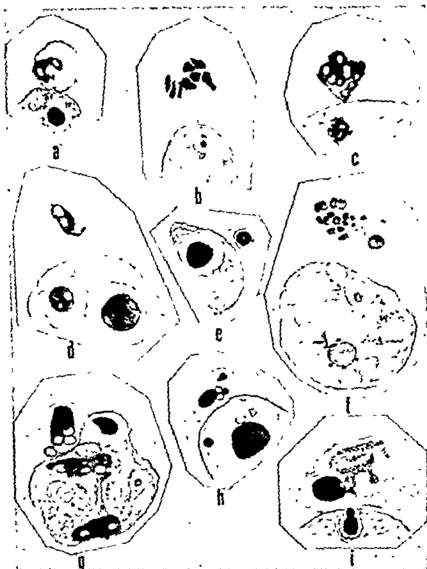


FIGURE 6. Extrusion of nucleoli (same tumor as in FIGURE 1). Original drawings in water color, taken from smears stained with methyl green and pyronin Y. Nucleoli in cytoplasm shown here are undergoing vacuolation, fragmentation, or other signs of disintegration. Descriptions are in the text. ×1100.

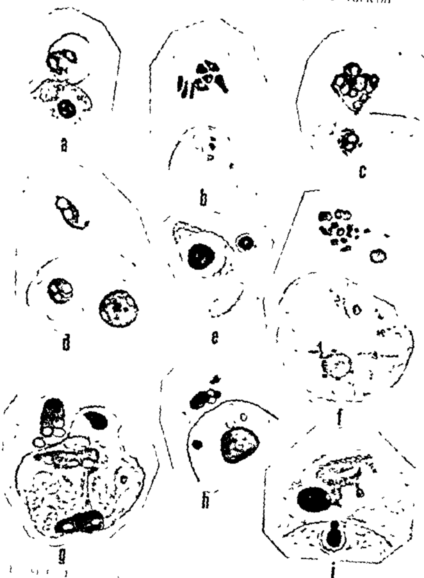
may be striking changes in morphology. Such changes might be induced by the action of cytoplasm on the extruded nucleoli. The development of excessive vacuolation as in FIGURE 6*a*, *c*, and *g* may be one of these changes. The configuration of the cytoplasmic nucleolus in FIGURE 6*a* shows the association



FIGURE 5. Extrusion of nucleoli (same tumor as in FIGURE 1) Original drawings taken from smears stained with methyl green and pyronin Y Nucleoli in cytoplasm as shown here are essentially unchanged Descriptions are in the text ×1000

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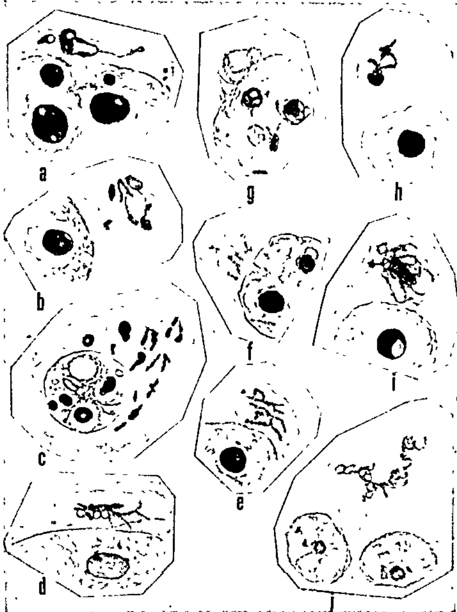


FIGURE 7 Extrusion of nucleoli (same tumor as in FIGURE 1) Original drawings in water color taken from smears stained with methyl green and pyronin Y Nucleoli in cytoplasm shown here are forming filamentous structures, in some, such structures were extruded directly from the nucleus Descriptions are in the text ×1150

of an alveolar mass, along with a vacuolated nucleolar mass. Such a configuration inside the nucleus would usually be accompanied by chromatin masses; however, in the cytoplasm such chromatin components are not found

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FIGURE 6*g* contains a considerable amount of nucleolar material consisting of two large, vacuolated masses joined together by what appears to be a thin

small alveolar

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The extruded nucleolus in FIGURE 6*e* is surrounded by a rim of methyl-green stained material This might be chromatin, on the other hand, this shell could be the remnant of the nuclear membrane, especially if part of it containing the nucleolus were pinched off from it. Of the many extruded nucleoli studied, this is the only instance seen in which the nucleolus in the cytoplasm was surrounded by methyl-green staining material

Following extrusion into cytoplasm, nucleoli may exist either completely or partially as filamentous configurations, such examples are shown in FIGURE 7. The filamentous configurations of the nucleolus in cytoplasm may result from direct extrusion of the filaments (FIGURES 1*m* and 5*c* and *d*) In other instances the filaments may be formed after the nucleoli are extruded (FIGURE 7*a*, *h*, *i*, and *j*) In all these examples it is clear that part of the nucleolus may still be recognized as a compact structure with occasional vacuolation in the main body The filaments themselves may be associated with vacuoles (FIGURE 7*d*, *h*, *i*, and *j*) The extruded nucleolar masses shown in FIGURE 7*c* could be the result of fragmentation following the formation of the filamentous configuration Several of the nucleolar filaments in cytoplasm suggest replication (FIGURE 7*a*, *d*, *e*, *g*, and *h*) The filamentous structure in FIGURE 7*g* is knotted and is a miniature version of the giant intranuclear filaments of FIGURE 3*n*, which are also knotted In each instance, the nucleolar masses are surrounded by chromophobic coronas No nucleolar-associated chromatin was evident in any of the cytoplasmic nucleolar filaments

The examples of nucleolar extrusion shown in FIGURE 8 are from cells in tissue culture of an omental metastasis of a carcinoma originating in the human endometrium Nucleolar extrusion is fairly common in these cells In some instances the nucleolus, while being extruded, is accompanied by additional nuclear materials, as in FIGURE 8*c*, *f*, *g*, and *m*.

In these cells, the first sign of extrusion is the appearance of a chromophobic zone even before any structures are actually extruded, as in FIGURE 8*a*, *e*, and *l*. Other examples show extrusion in progress (FIGURE 8*g*, *h*, *i*, *j*, *k*, and *l*) The extruded nucleoli or other derivatives of the nucleus are always surrounded by extensive chromophobic coronas.

In situ, the nucleoli in these cells are pleomorphic and frequently vacuolated, as in FIGURE 8*n* and *p* The vacuoles tend to be mainly marginal, although internal vacuolation does occur, as in FIGURE 8*o* and *q* These nucleoli,

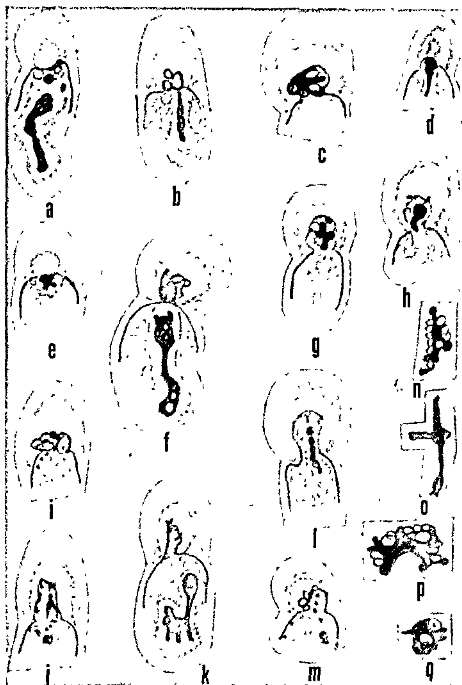


FIGURE 8 Extrusion of nucleoli (omental metastasis of human endometrial carcinoma, P & S No 42670) Original drawings in water color from tissue cultures stained with hematoxylin and eosin Descriptions are in the text ×1050

in situ, are always surrounded by chromophobic coronas, as in FIGURE 8*a*, *b*, *f*, *k*, and *l*

The following is a résumé of the observed variations in nucleoli during extrusion.

(1) The main bulk of the nucleolus is still in the nucleus, with a delicate network and some pyroninophilic vacuoles extruded into the cytoplasm The network is still attached to the nucleolar mass inside the nucleus (FIGURE 1*m*)

(2) A highly fragmented nucleolus, continuous with the nucleolonema in the nucleus, some solid nucleolar material, nucleolonemal or filamentous structures, and pyroninophilic vacuoles in the cytoplasm (FIGURE 5*d*)

(3) A partly extruded compact nucleolus with vacuoles Small solid nucleolar fragments in the cytoplasm (FIGURE 5*a*)

(4) A partly extruded nucleolar mass, with another similar mass already in the cytoplasm These masses are compact and without vacuoles (FIGURE 5*b*).

(5) A vacuolated nucleolus in the nucleus attached to an amorphous, vacuolated nucleolar mass extruded into the cytoplasm (FIGURE 5*c*)

(6) In some tumor cells the nucleolar body is extruded, while in others parts of the nucleus are pinched off and extruded with the nucleolus (FIGURE 8*b*, *c*, *d*, *e*, *f*, *g*, *h*, *i*, *l*, and *m*)

Over 50 examples of extruded nucleoli are illustrated in the figures The following list outlines the appearance of such nucleoli in the cytoplasm.

(1) Cytoplasmic or extruded nucleoli are clear-cut compact structures almost identical in appearance to nucleoli *in situ* The staining with pyronin is of the same intensity as shown by nucleoli *in situ* Peripheral borders are clear cut. Some nucleoli contain vacuoles, others do not In one binucleate cell, two nucleoli in the cytoplasm are almost identical in shape, size, and vacuolar pattern (FIGURES 1*l*, 2*r* and *s* and 5*f*, *h*, *k*, *l*, and *n*)

(2) Other extruded nucleoli are much more vacuolated, with somewhat irregular borders, probably indicating some disintegration Staining intensity with pyronin is similar to that of nucleoli *in situ* Several nucleoli have extensive marginal vacuoles Irregular shapes and diffuse peripheral borders of some of these nucleoli probably indicate the onset of degenerative changes (FIGURES 5*m* and *o* and 6*a*, *c*, *d*, *g*, *h*, and *i*)

(3) Fragmented nucleoli, apparently the disintegrative phases of previously compact nucleoli. Some of the fragments are vacuolated, others are attached to the vacuolar walls (FIGURES 5*a*, 6*b* and *f*, and 7*b* and *c*)

(4) Numerous nucleoli show filamentous, highly dendritic structures In some, this represents the nucleolar substance as it was extruded, while in others the unraveling or formation of nucleolar filaments must have occurred after extrusion Several examples were seen in which only part of the nucleolus had formed filaments These filamentous structures are occasionally associated with pyroninophilic vacuoles (FIGURES 5*d* and 7*a*, *d*, *e*, *f*, *g*, *h*, *i*, and *j*) In every instance, no matter how small the nucleolar fragment is in cytoplasm, it is surrounded by a chromophobic corona

In terms of nucleolar extrusion, there appear to be three principal kinds of nucleoli

(1) Solid nucleoli consisting mainly of *pars amorpha* On extrusion this

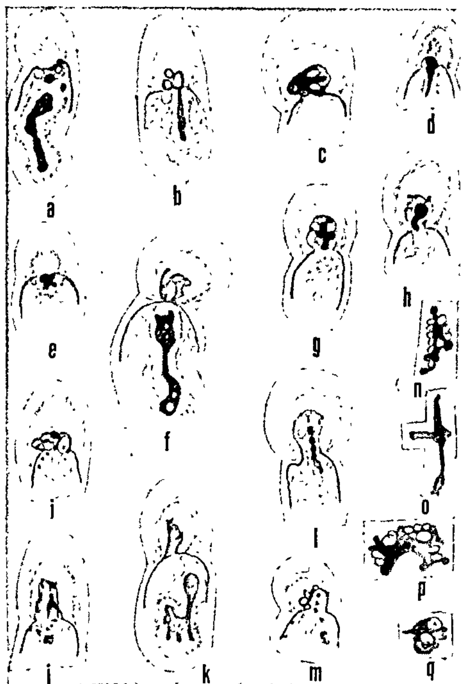


FIGURE 8 Extrusion of nucleoli (omental metastasis of human endometrial carcinoma, P & S No 42670) Original drawings in water color from tissue cultures stained with hematoxylin and eosin Descriptions are in the text ×1050

Srivastava[65] described the extrusion of nucleolar bodies in the oocytes of *Lumbricus*. In these cells, the globular or vacuolar bodies inside the nucleolus increase in size, apparently by the fusion of the smaller globules. When fully developed, the large globule is extruded into the nucleoplasm, and from there it passes through the nuclear membrane into the cytoplasm. These globules, either intranucleolar or cytoplasmic, do not become stained.

The extrusion of entire nucleoli occurs in the oocytes of *Holothuris*, as described and illustrated by Oka.[66] Usually, when the nucleolus is about to be extruded it is so heavily vacuolated that it resembles a morula; the actual extrusion involves the pinching off of a portion of the germinal vesicle membrane. After the extrusion or pinching-off process is completed the residue of the membrane is dissolved in the cytoplasm and a clear zone appears surrounding the nucleolus. The extrusion of nucleoli may be repeated in the same cell. On becoming embedded in the cytoplasm the nucleolus becomes more vacuolated, its outline becomes irregular and, as a result, it forms an amorphous mass that stains with acidic dyes such as eosin rather than with basic dyes, as it normally would inside the nucleus.

According to Chaudhry,[43] the nucleoli of teleost oocytes pass through the nuclear membranes into the cytoplasm as whole bodies. On reaching the cytoplasm, the extruded nucleoli may divide into smaller bodies and assume various shapes. The extruded nucleoli lose their staining properties and become only faintly colored. These nucleoli move toward the periphery of the cell and finally become lost in the cortical cytoplasm. At first, the size, shape, and staining properties of nucleoli either inside or outside the nucleus are exactly identical. In the eggs of *Salarias* as described by Eggert,[44] the nucleoli first appear in outpocketings of the nuclear membrane. Following evagination and further elongation of the pockets, constriction occurs and separation from the nucleus is completed.

During oogenesis of *Sacchobrancus*, *Clarias*, and *Anabas*, Narain[67] observed *extruded nucleoli in the cytoplasm. These nucleoli pass out of the nucleus* through the nuclear membrane as whole bodies without leaving a tear and finally dissolve away in the peripheral cytoplasm. During vitellogenesis in *Gasterosteus*, material from the nucleolus passes into the cytoplasm as granules or threads (Singh and Boyle[45]).

In tissue culture the nucleoli of mesenchymal chick fibroblasts migrate toward the nuclear membrane, and the amorphous substance is discharged into the cytoplasm (Lettré[37]). The extruded nucleolar material is surrounded by a clear zone. Lettré believed that the nucleoli in these cells consist of two parts: the amorphous portion that stains pink with pyronin, and the filamentous portion that stains with Feulgen regent or methyl green and, accordingly, is regarded as chromosomal in nature. On this basis, as mentioned previously, these filaments are not identical to the nucleolonema of Estable and Sotelo,[14] the latter being Feulgen negative. Lettré mentioned that the chromosomal part of the nucleolus, under normal conditions, remains intact in the nucleus, and new amorphous (pyroninophilic) material is accumulated.

In addition to the extrusion of nucleoli, for which stages showing the extrusion have been observed, there are other records of cytoplasmic nucleoli

type of nucleolus may remain intact, or it may acquire vacuoles and later become fragmented.

(2) Solid nucleoli that contain both the nucleolonema and *pars amorpha* On extrusion such nucleoli could remain intact, develop vacuoles, and become fragmented, or spin out filaments that later might also become fragmented

(3) Extrusion of filamentous structures with part of the nucleolar body remaining in the nucleus. Such nucleoli can be derived only from those bodies that possess the nucleolonema. In no instance are the extruded nucleoli or filaments from nucleoli associated with chromatin.

Other cells The extrusion of entire nucleoli or portions of them into the cytoplasm is by no means unusual. There are numerous instances occurring in vertebrate oocytes, for example, in the oocytes of *Amphiuma* and *Squalus* (Dodson[42]); various oocytes of marine teleostean fishes (Chaudhry[43]); eggs of *Salarios* (Eggert[44]); eggs of *Gasterosteus* (Singh and Boyle[45]); undifferentiated germ cells and young ova of *Xiphophorus* (Essenberg[46]); early oocytes of *Oryzias* (Oka[47]), and early stages of developing gametes of *Lophius* (Dodds[48]). In several instances, vitellogenesis has been related to nucleolar extrusion and the appearance of RNA in the cytoplasm

The extrusion of nucleoli into the cytoplasm of various cells in tissue culture is also reported Ludford[49] described this phenomenon in the fibroblasts from mouse tumors induced by tar applications In addition, Ludford[50] reported on the extrusion of nucleoli in fibroblasts of the kidney of the rat. In these cells, entire nucleoli may be extruded In others, only portions of nucleoli are extruded. Occasionally, a portion of the nucleus containing all or part of the nucleolus is pinched off, the contents of which may persist for a time as a secondary nucleus and then disintegrate. A slow ameboid movement of the extruded nucleoli was reported.

The discharged nucleolar material was surrounded by a vacuole, presumably the chromophobic corona In tissue culture, endothelial cells of the chick embryo also show nucleolar extrusion (Lewis[51]) In these cells the extruded nucleoli are surrounded by a "vacuolelike area"

Ludford[52] has described nucleolar extrusion in cells of the epididymis The phenomenon is believed to be related to secretory activity. The nucleoli, surrounded by a clear zone, persist for some time in the cytoplasm, only to become converted ultimately to small granules In this material the nucleolus is first acidophilic, then grows and becomes basophilic The basophilic nucleolus fragments and portions are extruded into the cytoplasm Lettré[37] has shown nucleolar extrusions in mesenchymal chick fibroblasts cultured in the presence of crystals of estradiol or methoxytoluquinone

Duryee and Doherty[53] described and illustrated the passage of nucleolar material from the nucleus to cytoplasm in the oocytes of *Triturus* Prior to this, Duryee[54] described the eversion of peripheral nucleoli through the nuclear membrane by treatment of the oocytes with dilute acids, for example Through such treatment the peripheral nucleoli could be induced to evert their contents through the nuclear membrane into the cytoplasm Multiple rounded structures were found in the surface of the nuclear membrane that corresponded to nucleolar remnants

The nuclei of the polynucleated cells are approximately 2 to 3 times the size of the lymphocyte nucleus and are, therefore, much smaller than the nuclei of the stromal cells that range from 5 to 7 times the size of the lymphocyte nucleus In addition, there is considerable difference in shape The nuclei of the polynucleated cells are more consistently simple in architecture and are usually ovoid in shape Occasionally, the relatively smooth contours will be interrupted by minor lobulations The nuclei of the stromal cells show a greater variability in shape, some being ovoid, others nearly spherical Then, too, these nuclei tend to considerable lobulation and may, indeed, be quite bizarre structures

Considerable differences in the nucleoli of the two cell types also exist. In the polynucleated cells the nucleoli are small and spherical and are generally compact, well-defined structures, no suggestion of nucleolar extrusion has been seen in these cells On the other hand, the nucleoli in the stromal cells are large (some are even larger than lymphocyte nuclei) and frequently vacuolated Many nucleoli have extremely bizarre shapes such as might, for example, be seen in FIGURE 3. In binucleated stromal cells there is usually a wide disparity in number, size, and shape of the nucleoli in the two nuclei

Unquestionably, nucleolar variability in terms of number, size, and shape reaches its peak in the stromal cells These are not unlike the nucleoli seen in tissue cultures of human ovarian tumors Angular, elongated, or irregular nucleoli have been previously reported from a study of Hodgkin's-diseased cells, among others, by Lopes Cardozo[62] Some of the nucleoli may have the shape of an exclamation mark

TABLE 1 summarizes the nucleolar numbers in polynucleated and stromal cells. Using as criteria nucleolar number and structure, it would seem unlikely that the polynucleate condition is achieved by any mechanism other than reasonably conventional karyokinesis On the whole, the nuclei and nucleoli in any one polynucleate cell are too similar to justify the conclusion that such a condition might be reached by the fusion of different cells.

As noted in TABLE 1, the number of nuclei per polynucleated cell tends to be six or more, with occasional cells seen in which over thirty nuclei have been counted The stromal cells are generally mononucleated The situation is reversed in reference to number of nucleoli per nucleus The polynucleated cells rarely have more than two nucleoli per nucleus, while the stromal cells rarely have less than four or five Another striking difference between these two cell types becomes evident if the nucleolar patterns are compared in binucleated and polynucleated cells Thus, 60 per cent of the nuclei in a polynucleated cell will have essentially identical nucleolar patterns in terms of size, shape, or number Approximately 40 per cent of such cells will manifest some differences, either in number (the most common difference) or in size or shape In the binucleated stromal cells, perhaps one cell in ten will have two nuclei with identical nucleolar patterns All others will show considerable variations in nucleolar patterns Furthermore, in binucleated stromal cells, even though the number of nucleoli might be the same, there is no conformity in either the size or shape of the latter There is, therefore, a much

For example, in *Tmesipteris*, Yeates[32] has seen nucleoli in the cytoplasm, apparently derived from the previous generation. These cytoplasmic nucleoli eventually disappear.

More recently, Heath[19] has prevented the disappearance of nucleoli during mitosis of chick fibroblasts in tissue culture with critical concentrations of cobalt chloride (0.01 to 0.015 mg/ml.). Although the nucleoli persist in cobalt ion treated cells, mitosis proceeds at the usual rate, and the distribution of RNA and DNA in both nucleus and cytoplasm remains unchanged. Following mitosis, the persisting nucleoli may become extranuclear, and then they may undergo slow random movements similar to chromosomal movements. The action of cobalt ions on the nucleoli may be a fixation of the RNA or the inhibition of a possible "nucleolar-dispersing enzyme." This effect cannot be produced by Ni, Zn, Cr, Mn, or Fe ions.

Nucleolar Variability in Lymph Node Cells (Hodgkin's Disease)

A series of cultures of lymph nodes from a patient with Hodgkin's disease (designated as series 218) was kindly supplied by A. Rottino of St. Vincent's Hospital, New York, N. Y. This series included cultures that were fixed with Zenker-formol at intervals ranging from 1 to 7 days of growth and then stained with the Giemsa mixture (Rottino[58]). The types of cells that emerge in the cultures include lymphocytes, eosinophilic and neutrophilic leukocytes, stellate wandering cells, and two types of relatively large cells.

One large cell type appears mainly during the first few days of culture and has a distinctive nuclear and nucleolar pattern. These cells, herein designated as polynucleated cells, have a tendency to develop many nuclei, and such cells may become giants. Cells similar to these have been seen in tissue cultures of Hodgkin's-diseased lymph nodes by Grand[59] and by Rottino.[58, 60] These cells are generally referred to as "multinucleated giant cells" by Rottino, and have also been called the Sternberg-Reed cells by Grand.

The second type reaches a peak in number of cells after 5 or more days of culturing. These cells, with a distinctive nuclear and nucleolar pattern, are of the fibroblast type, and were called giant stroma cells by Lewis.[61] Some of the nucleolar features of both the polynucleated and stromal cells are summarized in TABLE 1.

Table 1

	Polynucleated cells	Stromal cells
Number of nuclei per cell	6+	1 or 2
Average number of nucleoli per nucleus	2	4 or 5
Number of nucleoli in each nucleus of binucleated or polynucleated cell		
Same	60%	10%
Different	40%	90%
Nuclei with 1 nucleolus	33%	0%
Nuclei with 2 nucleoli	26%	14%
Nuclei with 3 nucleoli	14%	16%
Nuclei with 4 or more nucleoli	26%	70%

greater correspondence in the size, number, and shape of nucleoli in polynucleated cells.

A somewhat sharper breakdown shows that approximately 75 per cent of the polynucleated cells have three nucleoli or less per nucleus. On the other hand, the incidence of mononucleolate nuclei in the stromal cells is extremely rare. In these cells, approximately 70 per cent of the cells will have four or more nucleoli per nucleus

Of the two major types of large cells seen in tissue cultures of Hodgkin's-diseased lymph nodes, the stromal cells reveal the most dynamic features. These cells have large nuclei with enormous and unusually pleomorphic nucleoli. There is also much variation in nucleolar number and morphology, with 70 per cent of the cells possessing four or more nucleoli These cells undergo more frequent mitosis in tissue culture; also, there occur various atypical mitotic events such as chromosome bridges, tripolar spindles, and unequal distribution of chromosomes at anaphase, to name a few.

The extrusion of the nucleolus, or a fragment of it, by the nucleus is a fairly common event in stromal cells (FIGURE 9) In these cells it is generally a portion of the nucleolus that is extruded. This extruded fragment tends to be spheroidal in shape and compact. Its staining properties are identical to nucleoli *in situ*, or to the portion of the nucleolus remaining in the nucleus. In some instances, an outpocketing or evagination of the nuclear membrane is seen with a nucleolar portion in it. Without exception, the extruded nucleolar mass is always surrounded by a well-defined chromophobic corona.

Other evidences of unusual cellular activity include the ingestion of lymphocytes or other smaller cells by the stromal cells (also shown in FIGURE 9) In every instance the ingested cell is enclosed within a vacuolar zone that is sharply delimited by a clear-cut boundary between the vacuole and cytoplasm Freshly ingested lymphocytes maintain all their structural and staining features and cannot be distinguished from nearby uningested lymphocytes. Both cytoplasmic and nuclear features are clear The cytoplasm of the ingested lymphocyte first loses its staining properties, and this occurs before there is any appreciable pyknosis of the nucleus. Eventually, all that can be recognized from the ingested lymphocyte [illegible]
struc[illegible]
they [illegible]
the c[illegible]
optics Under phase contrast, the stained nucleoli appear as bright blue [illegible]

basic components in Giemsa's mixture and shows a high absorption, as indicated by the oscilloscope traces of single selected lines (FIGURE 9) A final feature is that almost invariably the ingested cell is quite close to the nucleus of the host cell and is separated by the thickness of the completely chromophobic vacuole in which the ingested cell is embedded



FIGURE 9 Stromal cell (Hodgkin's diseased lymph node, in tissue culture, St Vincent's Hospital No 218 24) Preparation was stained with Giemsa's mixture One vertical

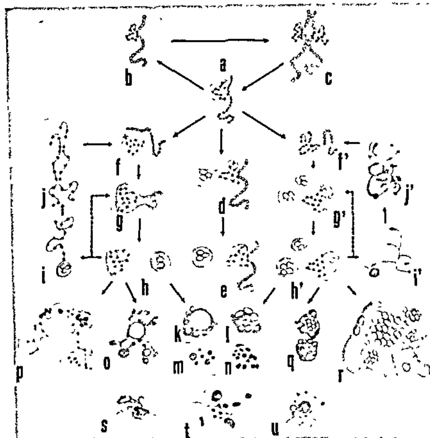


FIGURE 10. Chart [illegible]

(20) Variations in attachment of nucleoli to chromosomes, ranging from complete to partial separation of nucleolar-chromosomal complex from chromosome (FIGURE 10*f* and *f'*)

(21) Cytoplasmic nucleoli, entire and similar to nucleoli *in situ* (FIGURES 1*l*, 2*r*, and *s* and 5*e*, *f*, *g*, *h*, *k*, *l*, and *n*)

(22) Nucleoli in cytoplasm, heavily vacuolated, probably the onset of disintegration (FIGURES 5*m* and *o*, and 6*a*, *c*, *d*, *g*, and *h*)

(23) Cytoplasmic nucleoli, undergoing fragmentation and, possibly, disintegration (FIGURES 5*a* and *j*, 6*b* and *f*, and 7*b* and *c*)

(24) Nucleoli in cytoplasm, in filamentous configurations (FIGURES 5*d* and 7*a*, *d*, *e*, *f*, *g*, *h*, *i*, and *j*)

Types of Nucleoli in Neoplastic Cells

The following is a list of the various types of nucleolar lesions that have been seen in human and amphibian tumor cells. Reference is given to the illustration of the type, if included in this report.

(1) Hypertrophy, with tenfold to one thousandfold increase in volume (FIGURES 1*c* and *d*, and 2*c*, *d*, *e*, and *o*).

(2) Pleomorphism in shape, frequently coupled with hypertrophy and much vacuolation (FIGURES 1*e* and 4*a* to *o*).

(3) Multinucleolation: increased number of nucleoli, all of essentially the same size and shape (FIGURE 1*h* and *j*).

(4) Fragmentation. increased number of nucleolar masses of varying sizes and shapes (FIGURE 1*g*).

(5) Variations in basophilia, ranging from faint to intense staining with pyronin Y.

(6) Variations in eosinophilia, ranging from complete eosinophilia to none at all

(7) Development of vacuoles of various sizes and positions within the nucleolus (FIGURES 1*d*, *e*, and *f*, 2*b* to *s*, 4*a* to *o*; 5*f* and *l*; 6*g*; and 8*n*, *p*, and *q*)

(8) Appearance of small, highly refractile chromophobic inclusions, generally internal in position and much smaller than vacuoles

(9) Development of compact eosinophilic inclusions within basophilic nucleolus

(10) Appearance of Feulgen-positive structures within the nucleolus

(11) Excessive nucleolar-associated DNA, different in amount and structure from usual chromosomal DNA In some, entire nucleolus is closely surrounded by a layer of DNA, in others, small vacuolated masses of DNA are attached to the nucleolus Irregularly shaped masses of DNA, frequently large, may be attached to nucleoli Occasional nucleolar reticula (pyroninophilic) are closely integrated with DNA reticula (FIGURES 1*k* and 3*c* to *k*).

(12) Association of nucleoli with large nuclear inclusions consisting of clusters of pyroninophilic vacuoles of uniform size, also usually associated with DNA (FIGURES 1*k* and 6*a* and *g*)

(13) Formation of coarse and fine filaments and the unraveling of nucleolus into thin, frequently branching strands (FIGURE 3*a* to *p*).

(14) Modifications in pattern or staining of nucleolonema within compact nucleolus Structure ranges from single thread to a compact reticulum

icleated and

(16) Extrusion of entire nucleolus into cytoplasm (FIGURES 1*l*, 5*a* and *b*, and 8*c*, *g*, and *h*)

(17) Extrusion of filamentous structures (pyroninophilic) into cytoplasm [illegible] and 5*c* and *d*)

chromophobic zone (FIGURES 2*g*,

(19) Extrusion of eosinophilic or acidophilic substance into the nucleus and later into the cytoplasm (FIGURE 2*a*),

replication independent of mitotic mechanisms and can, therefore, reproduce
to

li,
and the nucleolus consists mainly of *pars amorpha* and associated substances, but it does not possess either the nucleolonema or the nucleolar organizer These nucleolar masses may be the pinched-off fragments from primary or autonomous nucleoli (FIGURES 10*e*, *h*, or *h'*). The derivatives of secondary nucleoli are frequently vacuolated or fragmented. The main bulk consists of the *pars amorpha* and associated configurations (FIGURE 10*k*, *l*, *m*, and *n*). Secondary nucleoli do not have the capacity for replication, since they lack both both the nucleolar organizer and the nucleolonema An apparent hypertrophy could be produced only if vacuolation occurred In instances where nucleolonema cannot be demonstrated within a nucleolar body, it is quite likely that this body is a secondary nucleolus

(4) *Intranuclear filaments* These filamentous structures are formed during replication of the autonomous nucleoli in interphase These may be accompanied by DNA in some instances and not in others (FIGURE 10*i*, *j*, *i'* and *j'*)

(5) *Extruded or cytoplasmic nucleolus* These are nucleoli extruded into the cytoplasm. Their configuration is either solid or vacuolated at first, although fragmentation and other changes may occur later The extruded nucleoli of this type might come from autonomous nucleoli without associated DNA, or they could come from secondary nucleoli Other extruded nucleoli may be filamentous and can be
ments are formed after e
only from the autono
found in the cytoplasm.

Nucleolar Cycles and Hyperactivity

The ensuing account considers the normal cycle of a nucleolus as well as modifications of this cycle that may be induced by endogenous or exogenous stimulatory agents

(1) *Normal reproductive cycle* During mitosis the primary nucleolus undergoes the cycle from *a* to *b* to *c* to *a'* and *a''* (FIGURE 10) The symbols *a'* and *a''* represent daughter cells resulting from mitosis If polyploidy or polysomic states involving the nucleolar chromsomes develop, then such a cell will gain in the number of primary nucleoli Certain stimulants (for example, metabolic demands or chemical agents such as thioacetamide) may induce a limited but reversible hyperactivity of the nucleolus through the sequence *a* to *d* to *e* (FIGURE 10) if budding of nucleoli occurs The budded portions would be classed as secondary nucleoli or fragments, depending on size. This type of hyperactivity requires the constant presence of the stimulus, otherwise, if the agent is withdrawn, the hyperactive response stops Under no circumstance can there be a reproductive phase of the primary nucleolus during the interphase

(2) *Abnormal reproductive cycle* This phase applies to autonomous nucleoli

General Discussion of Nucleoli and Nucleolar Lesions

The somewhat bewildering array of nucleolar structures shown by the nucleolar lesions eventually may fall into as orderly a sequence as that shown by cells undergoing mitosis Certain manifestations already may be pieced together in sequence. For example, a compact nucleolus is converted to a dendritic pattern by releasing the nucleolonema through the dispersion of the *pars amorpha* and loss of internal cohesion Intermediate stages, ranging from small coarse or fine filamentous extensions to complex dendritic configurations, were studied Hypertrophy and fragmentation are produced by the synthesis of nucleolar substances. Bizarre shapes follow hypertrophy and the generation of intranucleolar vacuoles, especially if the latter break through the nucleolar structure. The generation of pyroninophilic vacuoles by the nucleolus, the formation of large alveolar inclusions of similar architecture in the nucleus, and in the extrusion of such material into the cytoplasm represent another series of sequences

In the following discussion, an attempt has been made to integrate the various nucleolar patterns (see FIGURE 10). From this it is possible to reconcile the many variations in nucleolar behavior and structure. Furthermore, this scheme consolidates most of the presently available information on the structure of nucleoli, and clearly suggests a number of experimental approaches for further clarification of the role of the nucleolus in the cell. Other experimental approaches based on the generalized scheme may help in defining, fully and correctly, the role of the nucleolus in the neoplastic processes

In FIGURE 10 some of the diagrams and sequences are hypothetical, namely, the cycles starting with *f* and *f′* On the other hand, the terminal products, *i*, *i′*, *j*, *j′*, *k*, *l*, *m*, *n*, *o*, *p*, *q*, *r*, *s*, *t*, and *u*, are taken from the nucleolar lesions that were observed and described in neoplastic cells (see FIGURES 1 to 8)

Designation of Nucleolar Types

All the nucleolar configurations described in this report can be placed into one of five major categories The following is a description of each

(1) *Primary nucleolus* This type represents the nucleolar-chromosomal complex consisting of nucleolar organizer, nucleolonema, *pars amorpha*, and other substances normally associated with the nucleolus, and the nucleolar chromosome (FIGURE 10*a*) This unit has limited capacity for responding to hyperactive stimuli and requires constant application of stimulus to persist in the hyperactive state. The nucleolar-chromosomal complex represents the normal nucleolar configuration and can reproduce only through participating in normal karyokinesis

(2) *Autonomous nucleolus* In these nucleoli the nucleolar-chromosomal complex is disrupted. There may be two variants of this type of nucleolus One variant is the nucleolus without DNA, consisting of nucleolar organizer, nucleolonema, *pars amorpha*, and associated substances (FIGURE 10*f*) The other variant is a nucleolus with associated DNA, consisting of nucleolar organizer, nucleolonema, *pars amorpha* and associated substances, and chromosomal or chromatin residue (FIGURE 10*f′*) Both types have a capacity for

reproductive cycle	
a to *f* to *g* to *h* to *i* to *j* to *f* (to *g* to *h* . . .)	Leads to replication of nucleoli and possibility of further recycling either through *i-j-f*, or through *g-h-g* cycles

(4) *Atypical nucleolar cycle* (*with nucleolar associated chromatin*):

growth cycle:	
a to *f′* to *g′* to *h′* to *g′* (to *h′* to *g′*. . . .)	Leads to uncontrolled elaboration of *pars amorpha* and associated substances and, in addition, chromatin This cycle does not lead to an increase in number of autonomous nucleoli.
reproductive cycle.	
a to *f′* to *g′* to *h′* to *i′* to *j′* to *f′* (to *g′* to *h′*. . . .)	Leads to replication of autonomous nucleoli with possibility of further recycling through *i′-j′-f′* or through *g′-h′-g′* cycles

Activation of the Nucleolus

The nucleolus apparently can respond to a variety of stimuli by becoming hyperactive. The resulting hyperactivity may be either reversible or irreversible The difference between these two may now be focused on the stability of the nucleolar-chromosomal complex

(1) *Reversible hyperactivity* Hyperactivity is generally indicated by an increase in size (hypertrophy) plus a striking increase in output of nucleolar products. This type of hyperactivity is presumably induced by various metabolic demands, such as observed by Duryee[illegible] in amphibian oocytes or by chemical agents, such as the action of thioacetamide on liver cells (Kleinfeld[illegible]). These stimuli lead to increases in nucleolar size and in the amount of material released from the nucleolar body by vacuolar extrusion or budding. Whatever the action of the stimulus is, it does not dissociate the nucleolar-chromosomal complex The reversible hyperactivity is limited to *a*, *d* and, occasionally, *e* (FIGURE 10) The magnitude of the response is limited and requires continuous stimulation When the stimulus is withdrawn the nucleolar hyperactivity subsides as shown, for example, by removal of thioacetamide stimulation after nucleoli have been goaded to become larger in liver cells or to a lesser degree in kidney cells (Kleinfeld[illegible])

(2) *Irreversible hyperactivity* Other stimuli, either endogenous or exogenous, may bring about irreversible hyperactivity, providing these agents dissociate or disrupt the nucleolar-chromosomal complex, that is, from *a* to *f* or *a* to *f′* (FIGURE 10) Following this, various sequelae may emerge The precise response of the nucleolus would be modified by the properties of the stimulus

In any event, the hyperactivity of the autonomous nucleoli may be expected to continue after the stimulus is withdrawn Thus, the conversion of *a* to *f* or to *f′* is no longer reversible, since the normal nucleolar-chromosomal complex as a mutual regulatory mechanism no longer exists The nucleolus is autonomous because it possesses those components that permit growth or elaboration of various substances, and even replication during the interphase The initial action of a stimulus may be augmented by continuous application of

in which disruption of the nucleolar-chromosomal complex is induced either through the action of an agent or some accident within the cell. There are two variants of this reproductive cycle. In the first, the nucleolus is completely separated from its chromosome, as in FIGURE 10*f*. The first response to a hyperactive stimulus would be the sequence *f* to *g* to *h* (FIGURE 10), leading to hypertrophy, budding, and additional secondary nucleolar bodies. The nucleolar mass containing the nucleolar organizer, nucleolonema, and *pars amorpha* may recycle by the route *g* to *h* to *g* to *h*, and so on (FIGURE 10). The main end product of this cycle is an increase in either amount of nucleolar material or in number of secondary nucleoli. The detached nucleolar organizer contained by the nucleolus may induce filament formation (FIGURE 10*i*) and replication of the filament (FIGURE 10*j*) On reconstitution of the *pars amorpha* there will be an increase in the number of autonomous nucleoli; that is, reproduction of nucleoli during the interphase. Continued further hyperactivity may repeat the cycle *f* to *g* to *h* to *i* to *j* to *f*, and so on (FIGURE 10). There is no nucleolar-associated chromatin or DNA in this series.

The second variant involves the disruption of the nucleolar-chromosomal complex with part of a chromosome remaining attached to the nucleolar organizer (FIGURE 10*f'*) The stimulation of this complex to hyperactivity leads to the cycle starting with *f'* to *g'* to *h'*. Continued stimulation may recycle from *h'* to *g'* to *h'*, and so on. The nucleolar-associated chromatin in this series represents the residue from the chromosome that stays attached to the nucleolar organizer There may be an increase in the amount of chromatin [illegible]

(FIGURE 10*j'*) On reconstitution of the *pars amorpha*, there is an increase in nucleolar number (autonomous) and possible hyperactivity, such as from *h'* to *i'* to *j'* to *f'*

There is, therefore, the opportunity for the replication of both nucleolar bodies and associated chromatin during the interphase. Induced hyperactivity that leads to the dissociation of nucleolar-chromosomal complexes, either with or without nucleolar-associated chromatin, is essentially irreversible, principally because of the improbability that the nucleolar-chromosomal complex (FIGURE 10*a*) would ever be reconstituted if once separated

Résumé of Nucleolar Cycles

(1) *Normal reproduction during mitosis*

a to *b* to *c* to *a'*	*a'* represents the nucleoli in daughter cells resulting from mitosis

(2) *Normal elaboration of pars amorpha*

a to *d* to *e*	With limited or controlled elaboration of *pars amorpha* and associated substances

(3) *Atypical nucleolar cycle (no nucleolar-associated chromatin)*

growth cycle

a to *f* to *g* to *h* (to *g* to *h*)	Leads to uncontrolled production of *pars amorpha*, but without increase in nucleolar (autonomous) number

cleus should have the same number of nucleolar chromosomes and, accordingly, the same number of nucleoli. As described previously, this situation does occur, and one may see almost identical nucleolar patterns in the daughter cells. Yeates[72] also showed that binucleated cells of *Tmesipteris* not only had the same number of nucleoli, but that the fusion of nucleoli during telophase occurred simultaneously in the two nuclei.

In neoplastic cells, there are frequently great differences in number, size, shape, and distribution of the nucleoli in binucleated cells. In all probability, most of these differences are produced by the failure of mitosis to provide an equal distribution of nucleolar chromosomes in the daughter nuclei. There is no question that anomalies during mitosis can be seen in neoplastic cells. One of the more frequent incidents is the lagging chromosome or the chromosome bridge. If these chromosomes were also the ones carrying the nucleolar complex, then a monosomic, trisomic, asomic, or tetrasomic condition might occur, thus leading to reduced nucleolar numbers in one nucleus and increased nucleoli in the other.

Terminal Phases of Nucleolar Cycles

Most of the nucleolar lesions that have been described and illustrated in FIGURES 1 to 8 represent a terminal phase. A résumé based on the generalized scheme of nucleolar cycles (FIGURE 10) is presented below.

(1) *Nuclear masses lacking nucleolonema*
- (a) Usual intranuclear terminal phase:
  - Static, or enlargement of nucleolar body due to vacuolation, but no real increase in mass (10*k*, *l*).
  - Fragmentation, with or without vacuolation (10*m*, *n*)
- (b) Extranuclear terminal phase
  - Extrusion of nucleoli, as in 10*t* and 10*u*, where subsequent fragmentation or vacuolation, even disintegration, may occur in cytoplasm.

(2) *Nucleoli derived from f-g-h cycle*
- (a) Intranuclear phase
  - Growth and formation of large, bizarre nucleoli (10*o*).
  - Hypertrophy and pleomorphism may be followed by vacuolation (10*o*) or fragmentation (similar to 10*m*, *n*)
  - Formation of filaments (10*i*) and subsequent replication (10*j*)
- (b) Cytoplasmic phase
  - Extrusion of filaments from nucleus to cytoplasm (10*p*, *s*)
  - Extrusion of nucleolus, as in 10*u*, which may result in filament formation, increased vacuolation, fragmentation, or disintegration in cytoplasm
  - Extrusion of filament with part of the filament remaining in nucleus and another part in cytoplasm (10*p*). This arrangement ensures a nucleolar mass within the nucleus following consolidation of nucleolonema and reconstitution of *pars amorpha*, even though the equivalent of a nucleolus has been extruded into the cytoplasm. The fate of the nucleolonema in the cytoplasm is unknown.

the stimulus; that is, the passage from *f* to *h* or from *f'* to *h'* may be further goaded by excess stimulation. Reversal to normal status is improbable, since this would require reconstitution of the original nucleolar-chromosomal complex.

Factors That May Produce Variations in Nucleolar Number

The normal number of nucleoli in any cell depends on the number of nucleolar chromosomes contained within the nucleus. Thus, in cells of the human female there should be four nucleoli, since there are two autosomal nucleolar chromosomes and the two X chromosomes that are associated with nucleoli (Schultz and St. Lawrence[31]). Fusion, which may occur during telophase, could reduce the number to a minimum of one nucleolus (Matsuura,[26] Yeates[32])

In neoplastic cells, there are frequently many more nucleoli than could be accounted for on the basis of the euploid number of nucleolar chromosomes, such an increase in number can be produced in a number of ways. It should be emphasized that, in general, it is not possible to differentiate between primary, autonomous, or secondary nucleoli unless special methods are employed.

Nucleolar numbers may be increased by budding, as in *d* to *e*, *g* to *h*, or *g'* to *h'* (FIGURE 10). Replication of nucleolonema during the interphase, with reconstitution of *pars amorpha*, would increase nucleolar number, as in *i* to *j* to *f* to *i'* to *j'* to *f'* cycles. Budding, if followed by recycling and additional budding, would also increase the number of nucleolar bodies, as in *g* to *h* to *g* to *h* or *g'* to *h'* to *g'* to *h'*. The fragmentation of nucleoli, either with or without elaboration of nucleolar substance, would give an increase in number of nucleolar bodies, as in *m*, *n*, or the fragmentation of *o* (FIGURE 10).

Finally, an increase in the number of nucleolar chromosomes would increase nucleolar number. Aneuploidy, in which there is a polysomic condition specifically involving the nucleolar chromosomes, would account for abnormal numbers of nucleoli. The induction of polyploidy in the cell by any of the possible mechanisms (Hsu and Moorehead[64]) would also increase the number of nucleolar chromosomes. Thus, if the cycle from *a* to *b* to *c* goes back to *a* within the same cell, there would be a doubling of the nucleolar chromosomes. In all probability, aneuploidy with polysomic nucleolar chromosomes and polyploidy are perhaps the main agencies responsible for high nucleolar numbers in a cell (Gates[11])

The number of nucleoli in a cell may be reduced in several ways. The usual mechanism for this is the fusion of nucleoli, usually during telophase. Nucleoli may become extruded into the cytoplasm, as in *s*, *t*, and *u* (FIGURE 10), if these are modified, in the cytoplasm, to be unrecognizable, there would be an obvious reduction in nucleoli.

Nucleolar chromosomes may also be reduced in number, for example, during mitosis there may be an unequal distribution of nucleolar chromosomes. One cell may become trisomic while the other becomes monosomic. A similar effect could result from the failure of the normal recycling of the nucleolar chromosomes during mitosis, especially if the step from *b* to *c* fails.

The best cells for the study of variability in the number of nucleoli are the binucleated cells. If mitosis is correctly performed, then each daughter nu-

nucleolonema can become temporarily associated with a new set of chromosomes.

It would be highly unlikely that each set of anaphase chromosomes would acquire one of these nucleolonemata, however, at least one cell of the two might inherit the nucleolar components The next step would require the condensation of the nucleolonema to form a tuft and, following this, the accumulation of *pars amorpha* to produce a new autonomous nucleolus Ordinarily, the *pars amorpha* begins to accumulate at telophase before the new nuclear membrane is reconstituted (FIGURE 1a)

To what extent can nucleolar bodies lacking the nucleolonema and nucleolar organizer (FIGURE 10k, l, m, and n) be transmitted to the next generation of cells? The chances are probably zero. The reason is that the main bulk of the nucleolar body would become dispersed during the onset of mitosis, since it consists mainly of *pars amorpha*. The nucleolar body, however, could not become reconstituted during telophase, since there is no nucleolonemal tuft onto which the *pars amorpha* could accumulate. The dispersed *pars amorpha* would most likely become scattered in the cytoplasm, although some of this substance might be transmitted to both cells just as most other cytoplasmic inclusions and components are The most reasonable interpretation is that the nucleolar bodies lacking the nucleolonema are the end of the line and cannot be transmitted to successive cellular generations The same fate probably awaits the extruded nucleolar bodies, unless they may still have a nucleolonema and nucleolar organizer capable of surviving in the cytoplasm If so, then these could possibly be transmitted in the way suggested for nucleoli derived from the *f* or *f'* cycles

Transplantation of Nucleoli from Cell to Cell

There are many questions to be answered. Are the nucleoli the key to the problem of the neoplastic cell? Are nucleolar lesions directly involved in malignancy, or are they the result of malignancy? To what extent is the nucleolar-chromosomal complex one of the basic regulatory mechanisms in the cell? What is the essential cytochemistry of the nucleolus? What are the real functions of the nucleolus, whether it be in a normal cell or in a malignant one? Certainly, the best and, probably, the only way of approaching answers [illegible] velopment of various micrurgical techniques for transplanting subcellular particles With these methods (Kopac[65-67]), nucleoli have been transplanted from one cell to another The implantation of inclusion bodies from neoplastic cells into normal cells is another approach

As described by Kopac,[65, 66] nucleoli can be removed from the nucleus of one cell and placed into the cytoplasm of another cell with siliconized micropipettes and semiquantitative volumetric controls Micropipettes with apertures of about one micron are suitable for removing pieces of the nucleolus, while larger micropipettes are necessary for transplanting nucleoli. The inclusion bodies, which may develop as a consequence of viral infection in the nucleolus, the

(3) *Nucleoli derived from f'-g'-h' cycle.*

(a) Intranuclear phase:

Growth and formation of large, frequently bizarre nucleoli (10*r*) with extensive hypertrophy and pleomorphism

Nucleoli form pyroninophilic, vacuolated inclusions that are always associated with DNA and large RNA masses (10*r*)

Formation of filament (10*i'*) and replication (10*j'*) These filaments are always associated with DNA, and there is a frequent suggestion of duplication of DNA (10*j'*) as well. Structure of DNA masses is too irregular to be considered, *per se*, as duplication of chromosomes.

(b) Cytoplasmic phase.

Extrusions of nucleoli or nucleolar filaments associated with DNA do not occur. Extruded filaments may be associated with DNA in the nuclear phase, but DNA either is not extruded or else is destroyed as fast as it reaches the cytoplasm.

Transmission of Nucleolar Characteristics from Cell to Cell

problem. Obviously, if the nucleolar-chromosomal complex is intact (FIGURE 10*a*), then propagation of the nucleolus goes through the normal mitotic cycle, from *a* to *b* to *c* to *a'*, with reorganization of nucleoli occurring during the telophase. Thus, nucleoli that have been modified to go through the progression *a* to *d* to *e* can be propagated and passed onto the next generation.

The exception to the propagation of an acquired hyperactivity might occur if the action of the stimulus were focused primarily on the *pars amorpha* This fraction apparently is not directly continuous from one generation to the next in the same way the nucleolonema, the nucleolar organizer, and the chromosome are

On the other hand, what are the chances of propagating nucleoli derived from the *f* or *f'* series? If the nucleolus from the *f'* series is attached to a chromosomal fragment that includes the kinetochore, there is a fairly good possibility that the unit might participate normally in the mitotic cycle This is generally true whenever any chromosomal fragment carries the kinetochore Chromosomal *fragments without the kinetochore may encounter a difficulty,* if not an impossibility, in entering into normal mitotic events.

Nucleolar derivatives of the *f* series or *f'* series, in which the kinetochore is not a part of the chromosomal fragment, could be propagated from cell to cell, but only in a haphazard manner These nucleoli would, in all probability,

nucleolonemal strands become extended there is a possibility of becoming entangled with some of the chromosomes, and then these may be pulled to one of the poles during anaphase along with the chromosomes In this way the

nucleus, or the cytoplasm, can also be transferred to any part of the same or different cells with micropipettes and precisely controlled microinjectors. A scheme for designing and recording experiments has been devised especially for transplanting nucleoli and inclusion bodies (Kopac[65]).

Transplantation of Nucleolus into Cytoplasm

The living cells used for these experiments were those of the Lucké adenocarcinoma of the frog kidney grown in a fluid medium in tissue culture. Transplantations of the nucleoli shown in FIGURE 11 were made by micropipette transfer using the television-micromanipulator-oscilloscope unit (Kopac[66]). Approximately 12 hours after the nucleoli were transferred, the cells were fixed in cold methanol. They were then stained with methyl green and pyronin Y according to the method of Long and Taylor[7] to bring out differentially structures containing both DNA (stained green) and RNA (stained rose-pink). After 12 hours in the cytoplasm of another cell, the experimentally transferred nucleoli have the same color as the nucleoli *in situ*.

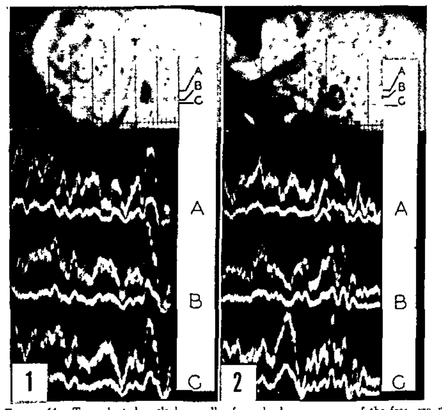
The photographic records of the nucleoli were taken from the video screen with a Polaroid camera. The pictures are usually triple exposures so that the positions of the lines selected for analysis can be seen. The oscilloscope traces of the video lines selected for analysis are positioned and the sweep time adjusted in order to register the key vertical positions of the traces with the structures shown in the cells. A large amount of data, especially the detection and measurement of changes in nucleoli, can be derived by this method of recording video images and oscilloscope traces.

The transplanted nucleolus shown in FIGURE 11 (2) has developed a large, prominent central vacuolar zone that is clearly indicated by both the video image and the oscilloscope traces, in particular lines B and C. It is evident *that the density of the transplanted nucleolus is almost as high as that of the nucleolus in situ*. Both nucleoli are surrounded by chromophobic coronas.

In another experiment (Kopac[67]) the transplanted nucleolus did not form vacuoles, and its optical density was approximately equal to the density of the nucleolus *in situ*. Both of these nucleoli were surrounded by chromophobic coronas, with the more prominent corona surrounding the transplanted nucleolus.

Nucleoli, following transplantation and approximately 12 hours' sojourn in a new environment, such as the cytoplasm of a different cell, may or may not become vacuolated. At the present time there is no explanation why some nucleoli are vacuolated following transplantation and others are not. It is clear that the appearance of the vacuoles in the transplanted nucleolus need not necessarily be the result of transplantation per se. On the other hand, all transplanted nucleoli become surrounded by the chromophobic corona. The transplantation of cytoplasm from one cell to another (frog liver) does not cause any chromophobic zones to develop (Kopac[68]), thus indicating that transplantation, per se, is not responsible for the chromophobic coronas associated with transplanted nucleoli.

In general, nuclei are highly susceptible to mechanical puncture. This effect was described by Chambers and Fell,[69] who showed that the nuclei of



zone In this instance, the maximum density of the transplanted nucleolus is almost as high (line C) as the nucleolus *in situ*

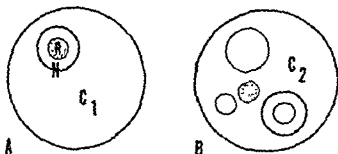


FIGURE 12 Diagrams of operation for comparing transposed and transplanted nucleoli in a living cell In these diagrams, n is the nucleolus, N the nucleus, C_1 the cytoplasm of cell 1, and C_2 the cytoplasm of cell 2 The donor cell (A), using symbolic notation, is $n_1 e N_1 g C_1$ (Kopac[66]) In (B), the cell is shown after a nucleolus was transferred from the nucleus to cytoplasm, followed by transplantation of a nucleolus from cell 1 Thus, after transposition of the nucleolus (nonstippled) to cytoplasm, the experiment is designated $n_2 e C_2 - N_2 e (n e N)_2$ Following transposition of nucleolus 2, the nucleolus (stippled) from cell 1 is transplanted, to give $n_1 e n_2 e g C_2 - N_2 e (n e N)_2$ The last statement states that the nucleoli from cell 1 and cell 2 are a subgroup in the cytoplasm of cell 2, but not of the donor nucleus (N_2) nor of the intact nucleus $(n e N)_2$ It is necessary to use a binucleated cell in order that the cell may survive after one of its nuclei has been damaged by removal of the nucleolus

multinucleated cells, as described in the previous section In FIGURE 12A and B, an operation is diagrammed that permits the comparison of a transposed nucleolus with one that has been transplanted This is the only type of experiment that allows one to evaluate the possible effects of the cytoplasm on a nucleolus or vice versa, since this procedure supplies the same environment for both nucleoli The method requires a binucleated cell so that the removal of the nucleolus, with subsequent destruction of the donor nucleus, will not lead to death of the cell. Experiments of this type will help answer the question whether cytoplasm promotes the vacuolation frequently seen in both extruded and transplanted nucleoli

It is, unfortunately, impossible to implant a nucleolus into the nucleus, however, a practical approach has been designed that may yield the same result as if the nucleolus had been initially implanted into the nucleus Accordingly, the possible way of introducing a nucleolus into a nucleus is an indirect one, in that the nucleolus can be placed near one of the chromosomal masses during late anaphase or early telophase, prior to reconstitution of the new nuclear membrane Under these conditions there is a good possibility that the transplanted nucleolus will be enclosed by the new post-telophase nuclear membrane Reference to FIGURE 16 will show that, at this time, the newly formed nucleoli following mitosis are surrounded by the telophase chromosomes Later, the new nuclear membrane is formed around this chromosomal regrouping

The operation is represented diagrammatically in FIGURE 13A and B The origin of the nucleolus, using the symbols described previously by Kopac,[66] is indicated in the diagram (FIGURE 13A) In this instance, the nucleolus comes from a multinucleolate nucleus Between prometaphase and early telophase,

chick fibroblasts, in tissue culture, are easily and irreversibly damaged by puncture with a microneedle. A mononucleated fibroblast will disintegrate completely if its nucleus is punctured The nucleus first becomes coagulated; then a granular precipitation is produced in the cytoplasm, thereby killing the cell in a short time. In binucleated fibroblasts, the punctured nucleus also coagulates, but the disintegrative changes in the cytoplasm occur only near the injured nucleus. The intact second [illegible]

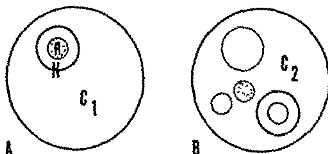
field shown by the photograph, this cell contained several nuclei, so that the cell lived despite the destruction of one of them. The nucleolus depicted in the picture was removed from the nearby nucleus at the time of interphase. The nucleus shows obvious signs of deterioration, such as the appearance of a coagulated mass that has shrunken away from the nuclear membrane. There is almost complete loss of staining of the nuclear residue by methyl green Essentially, no changes are evident in the cytoplasm. The nucleolus maintained its density and pyroninophilic properties, although an incipient vacuole was developing, as shown in the video image and selected line, C. The nucleolus is surrounded by a chromophobic corona. Even though the interphasic nucleolus is now in a new environment (the cytoplasm of the same cell) it has survived the operation, while the nucleus has not

There is no explanation, as yet, for the chromophobic corona that is so frequently associated with nucleoli, either *in situ*, extruded, or transplanted. Furthermore, these coronas can be seen in living cells by phase-contrast optics or even by transmitted light and, consequently, are not necessarily a fixation artifact There is a fairly good chance that the substance making up the corona comes from the intranucleolar vacuoles The cytochemistry of the chromophobic corona is now under investigation, Mateyko and Kopac,[70] have found that the corona resists most staining techniques, and that the substance within it contains carbohydrates The cytochemistry of the substance within the nucleolar vacuoles will probably pose an interesting problem. So far, the only cytochemical studies reported on nucleolar vacuoles are by Macary[14] and these are unable to state anything positive Nevertheless, the vacuolar material is capable of inducing cytochemical changes that render nucleoplasm or cytoplasm unstainable by such dyes as methyl green, pyronin Y, hematoxylin, eosin, or phloxine

Transplantation of Nucleolus into the Nucleus

The extremely desirable operation of transplanting a nucleolus from one cell to the nucleus of another cell is one of the most delicate operations, and the survival of cells following this operation is rare indeed The reasons for this are the susceptibility of nuclei to mechanical puncture, and the necessity for puncturing the nuclear membrane of an interphasic nucleus in order to implant the nucleolus

Even the important operation of transposing (Kopac[66]) a nucleolus from the nucleus to the cytoplasm of a cell can be accomplished only in binucleated or



[illegible] *for comparing transposed and transplanted nucleoli*

[illegible]

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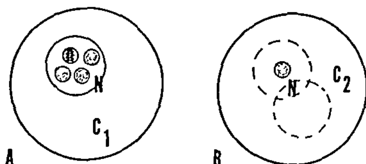
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Although the technique of subcellular transplantation has been advanced to the point that nucleolar transplantation is possible, and although, in addition, rapid scanning methods are available for analyzing changes in some of the properties of the transplanted nucleolus, most of the important biological effects cannot be answered until better methods are available for the propagation of experimentally modified cells in tissue culture. There is already some progress in this direction, including the development of a special chamber adaptable for both micrurgy and tissue culture. In addition, some of the methods for culturing relatively few cells and producing clones therefrom have been developed and tested by Puck and his associates.[71] With these improvements in procedure, and with the background of information presented in this report, there should soon be an answer to the fundamental question: What is the role of the nucleolus in the cancer process?

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The dotted lines

there is no nuclear membrane; consequently, the dotted circles in FIGURE 13B represent the fields now occupied by chromosomes and later become the sites of new nuclei formed during telophase.

An important time factor is involved The new nucleolus should be implanted near the chromosomes at the instant the new nucleoli would ordinarily make their appearance (see FIGURE 1*a*). By this time the anaphase is completed and the telophase is commencing The *pars amorpha* is elaborated at this time, thus making it unlikely that the *pars amorpha* of the implanted nucleolus would be modified Usually, the nucleolus is the first prominent structure to make its appearance after the chromosomes have reached their polar position (end of anaphase) and begin to regroup In chick fibroblasts, for example, the nucleolus becomes reconstituted several minutes before the new nuclear membrane is formed

important is the fact that no nuclear membrane need be punctured Furthermore, the nucleolus is actually implanted into the cytoplasm, and the temporary survival of a nucleolus in cytoplasm is assured on the basis of transplantations already performed

Assuming successful "adoption" of the transplanted nucleolus, it will be possible to begin to get answers to several questions that have been raised, especially in reference to the transmission of acquired nucleolar characteristics to successive generations of cells For example, will the *pars amorpha* of ... ive nucleoli "disappear"? ... next generation if it lacks

ADVANCES IN THE KNOWLEDGE OF THE EOSINOPHIL IN RELATION TO ANTIBODY FORMATION*

By Robert S Speirs

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In an earlier review published by The New York Academy of Sciences in 1955,[61] it was reported that quantitative hematological techniques could be used to determine the cellularity of fluids, such as the peritoneal fluid, which accumulate in different areas of the body. This approach made it possible to correlate local (peritoneal fluid) and systemic (blood) changes in leukocytic and reticuloendothelial cells following intraperitoneal injections of hormones, drugs, antigens, and other such substances It was noted at that time that following repeated injections of antigenic material there was an accumulation of eosinophils between the second and the tenth day after the challenging injection. These experiments suggested the existence of a relationship between the function of eosinophils and immunity responses This paper reports further studies of changes in eosinophils during immunological reactions

An accumulation of eosinophils around parasites or after injections of parasitic extracts has been repeatedly reported in the literature[5, 6, 11, 31, 32, 52, 66] It seemed advisable first to determine whether quantitative measurements of this response could be made, and then to determine some of the factors that change the magnitude of the response Extracts of the roundworm *Oxyuris* were prepared and injected intraperitoneally into mice Animals were autopsied at various times prior to and after the injection, peritoneal fluid was withdrawn, and chamber counts were made to determine the total cellularity Smears were also made for differential counts The techniques used are described elsewhere.[65, 66]

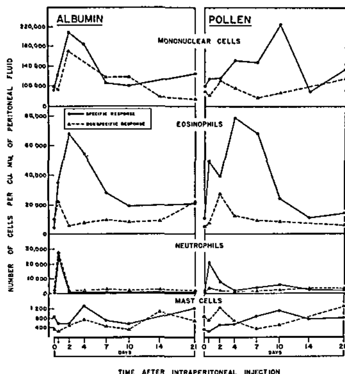
The results obtained from a typical experiment are illustrated in FIGURE 1. It may be seen that a slight increase in the number of eosinophils occurred after the initial injection of either a saline extract or a suspension of the worms On the other hand, if the animals were pretreated with a series of five subcutaneous injections of either material, a challenging intraperitoneal injection produced a marked eosinophilia Similar results following injections of extracts of the roundworm *Ascaris* were reported earlier [61]

These experiments indicate that, although a local eosinophilia can be produced by injection of certain protein materials, the magnitude of the response depends in part on previous exposure of the animal to that material

As we continued to improve our techniques we found that much of the variation brought about by stress could be eliminated by first adrenalectomizing the animals, then sensitizing and challenging them [59] Responses similar to those observed in intact mice were obtained following the injection of a wide variety of antigenic substances FIGURE 2 summarizes the results obtained

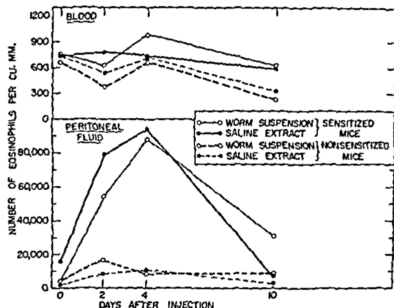
* The experiments that led to this hypothesis were carried out during the past six years in three different laboratories The work was supported primarily by grants from the Public Health Service, Bethesda, Md, and by funds from the Atomic Energy Commission, Washington, D C

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to the antigen These data confirm and extend the results published earlier by Biggart,[7] Ringoen,[39] Schlecht,[48] and others

It was of interest to determine whether the accumulation of eosinophils is brought about by the presence of parenteral proteins per se, or whether a specific type is essential Experiments were therefore designed to determine the eosinophil response to an injection of protein material identical to that of the recipient animal Lyophilized extracts of spleen were prepared from tissue taken from mice of three inbred strains and were used to sensitize and challenge other mice of the same strains and mice of different strains,[8] the results are summarized in FIGURE 4 It may be seen that single or repeated injections of spleen taken from donor animals genetically identical to the recipients produced only a slight temporary increase in eosinophils On the other hand, repeated injections of spleen extracts from animals that differed genetically

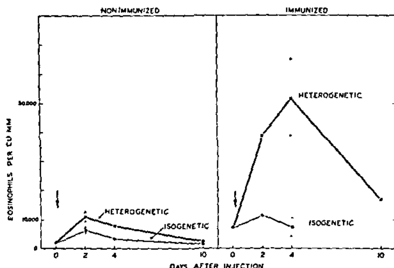


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average of at least six mice

following injections of ragweed pollen extract and bovine serum albumin Animals that were sensitized and challenged with the same antigen showed a much greater and more prolonged accumulation of eosinophils in the peritoneal fluid than did similar mice that had been injected with an antigen differing from the sensitizing antigen The mononuclear cells (histiocytes) also increased in number after the reinjection of a specific antigen, but this response was found to vary, depending on the antigen injected The number of neutrophil and mast cells was not consistently different in the specific and nonspecific response

In order to determine the relationship of sensitization to the eosinophil response, we compared the 4-day response in animals that had received different numbers of sensitizing injections at weekly intervals The results obtained are shown in FIGURE 3. It may be seen that, with repeated sensitizing subcutaneous injections, there was a progressively greater accumulation of eosinophils following each challenging intraperitoneal injection up to the fifth On the other hand, there appeared to be no consistent response pattern in the number of mononuclear cells These and other experiments indicated that [illegible] extracts, ragweed pollen extracts, tetanus [illegible] erythrocytes, foreign serum, or serum [illegible] There is a relatively slight eosinophilia in response to the first injection, and a progressively greater and more prolonged accumulation of eosinophils following each successive exposure of the animals



[illegible]
of eosinophils occurred with the isogenetic extracts, while marked response occurred with the heterogenetic extracts.[8]

Figure 5 illustrates changes in serum antitoxin titers as well as in the eosinophil response following a challenging injection of tetanus toxoid in tetanus-immunized mice. It may be seen that a challenging injection of tetanus toxoid produces a local eosinophilia at the site of injection prior to or simultaneous with the increase in serum antitoxin titers. It may also be noted that the change in local eosinophil concentration is reflected in the blood, spleen, and bone marrow. The data obtained show no evidence that any of the eosinophils seen in the peritoneal fluid are produced locally. All the cells responding to the antigen injection are segmented, fully mature cells. On the other hand, there is an immediate decrease in the number of blood and splenic eosinophils and a progressive increase in the number of the immature cells of the bone marrow. By the sixth day after the challenge these initial changes are followed by a corresponding increase in the number of segmented forms in the bone marrow, blood, and spleen.

These data confirm the concept that eosinophils responding to the antigen come from the circulating blood. After a challenging injection the level of eosinophils in the blood is maintained first by splenic eosinophils and later by the differentiation of stem cells in the bone marrow.

A series of studies was undertaken to determine the fate of the eosinophils that had accumulated in the peritoneal cavity following a challenging injection. Although our studies are not yet complete, preliminary results indicate that there is extremely little phagocytosis of eosinophils in animals that have re-

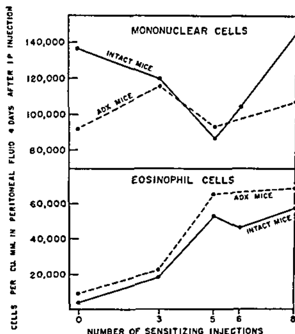


FIGURE 3. The influence of varying degrees of sensitization on the local mononuclear and [illegible]

from the recipients produced a prolonged and greater accumulation of eosinophils

These experiments indicate that the eosinophil response is not merely the result of a local inflammation brought about by protein injection, but the response is part of the cellular reaction of the animal to a foreign protein The eosinophil response must therefore be considered part of the immunity mechanism of the animal

The relation of eosinophils to immunity responses is further indicated by the fact that repeated injections of the same antigen must be made in order to obtain a maximum eosinophilia These injections should be spaced at intervals of from 5 to 25 days[21, 40] Furthermore, the eosinophil response reported here is not only characteristic of the peritoneal fluid, it is characteristic of the fluid at any site of penetration of an antigen Accumulations of eosinophils have been reported around the antigen whether it was introduced intraperitoneally, subcutaneously, intravenously, or via the digestive or the respiratory tracts These cells also accumulate in lymph nodes draining an area of injection or infection and in the spleen following an intravenous injection of foreign material[2, 11, 16, 22, 29, 40, 49, 67]

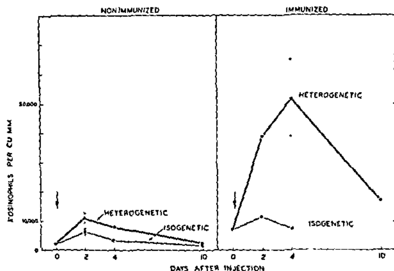


FIGURE 4 [illegible] heterogenetic extracts[8]

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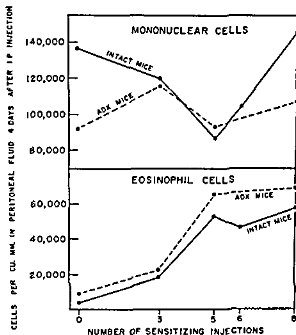


FIGURE 3 The infl[illegible]

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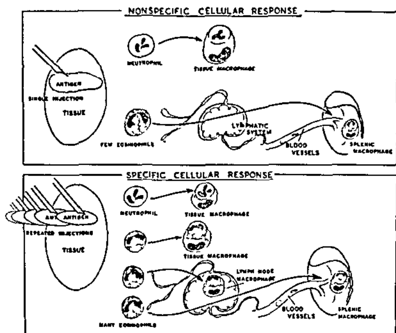


FIGURE 6 illustrates diagrammatically the changes observed at the site of antigen injection. If the antigen is injected for the first time, the response is considered nonspecific If the animal has been previously sensitized or immunized to the antigen a different response occurs, which is referred to as a specific response In the nonspecific response there is a migration of neutrophils and a few eosinophils out of the blood vessels and into the peritoneal fluid Although there is some local phagocytosis of the neutrophils, very little or no phagocytosis of eosinophils is normally seen Most of the cells have apparently been washed out of the peritoneal cavity and are later phagocytized in the lymph nodes and spleen In the specific response, the neutrophil response remains essentially unchanged, but there is a much greater magnitude of response by the eosinophils These eosinophils are phagocytized by macrophages, both locally and in the draining lymph nodes and spleen These macrophages are part of the reticuloendothelial system and originate not only from mitosis of local histiocytes, but presumably also from lymphoid cells migrating from the blood vessels and surrounding tissues[34, 61]

The eosinophil response to a challenging injection of antigen can therefore be divided into four stages

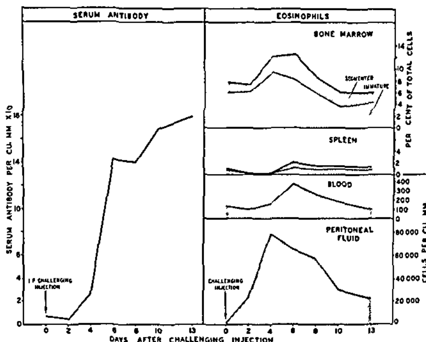


FIGURE 5 Serum antibody titers and eosinophil responses at various periods following a challenging injection of tetanus toxoid At least 19 mice were autopsied for each day represented on this chart Antibody titer represents the minimum lethal dose (MLD) of tetanus toxin neutralized by 1 cu mm of serum This was assayed by partial neutralization with toxin and serial dilution of the remaining antitoxin with a standard toxin Young mice were used for the assay

ceived an antigen injection for the first time It should be noted, however, that cortisone injections or stress can produce an increase in phagocytosis of eosinophils in these animals[10, 34, 35, 37, 43] On the other hand, examination of the peritoneal fluid smears of immunized mice indicates that there is a great deal of phagocytosis of eosinophils by mononuclear cells even without stress or cortisone injections This could be seen even in the counting chambers and was especially evident on the fourth and sixth days after a challenging injection Moreover, many of the eosinophils observed within the macrophages appeared to be in excellent condition morphologically, which is in contrast to those observed by Fruhman[17] and by Poel[37] after stress or cortisone.

It seems probable that, following the first injection of antigen, the eosinophils are rapidly washed away from the site as a result of the inflammatory edema These cells are presumably phagocytized later in the spleen and other tissues containing reticuloendothelial cells.[10, 16, 33, 40] In the actively immunized animal there are local antigen-antibody reactions, resulting in the fixation of antigen[29] and followed by a greater accumulation of eosinophils over a prolonged period of time The macrophages engulf not only particulate antigen, but neutrophil and eosinophil cells as well The fate of the eosinophil is therefore similar to that ascribed to the neutrophil

TABLE 2

LOCAL EOSINOPHIL RESPONSE FOLLOWING AN INJECTION OF TETANUS TOXOID IN ACTIVELY IMMUNIZED MICE AND MICE PASSIVELY IMMUNIZED WITH RAT ANTISERUM*

Type of immunization	Total No of mice	Challenging injection IP	Eosinophils† per cu mm
Nonimmunized (Animals injected with normal rat serum)	41	0 2 ml Tetanus toxoid	6626 ± 1682
Passively immunized‡ (Animals injected with from 0 1 to 1 0 ml rat antitoxin)	56	0 2 ml Tetanus toxoid	6395 ± 975
Actively immunized (Animals previously injected with tetanus toxoid antigen)	40	0 2 ml Tetanus toxoid	70739 ± 8977

* Reproduced by permission of *RES Bulletin* [64]

† Counts taken 4 days after challenging injection

‡ Antiserum injected from 1 hour to 6 days prior to challenging injection

rats given repeated injections of tetanus toxoid [53] Again the eosinophilia observed was not any greater than that observed in animals receiving injections of normal rat serum or of nonspecific toxoid, as can be seen in TABLE 2. Finally, we obtained large amounts of mouse serum to use for passive immunization of our animals (FIGURE 7) We tried various combinations of antigen and antibody, not only in normal mice but also in adrenalectomized mice, in which sensitivity reactions are much more likely to occur, but we were not able to produce an eosinophilia On the other hand, if the toxoid was first combined with the antitoxin and then injected into actively immunized animals a local eosinophilia was obtained Our data indicated that either the toxoid or the

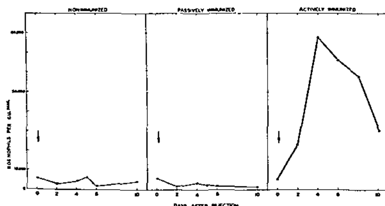


FIGURE 7. Local eosinophil response following a challenging

(1) An immediate, local inflammatory stage involving margination, diapedesis, and migration of cells out of the blood vessels and into the area of injection, or fixation of the antigen This stage occurs immediately after the injection and lasts for approximately six days.

(2) A stage of reaction of eosinophils with the antigenic material The eosinophils are chemotactically attracted from the blood vessels into the tissue spaces containing the antigen Phagocytosis of the antigen is seldom seen, but some reaction must occur between the eosinophil and the antigen

(3) A stage during which there is phagocytosis of the eosinophils by cells of the reticuloendothelial system. This stage begins between the second and fourth days after injection and lasts for 10 days or more

(4) A proliferative stage in which new eosinophils are formed in the bone marrow This stage appears to reach a peak between the fourth and sixth days after the challenging injection.

It can be observed in FIGURE 5 that antibodies were present at the time of antigen injection and that they increased in titer between the fourth and sixth days after the challenging injection It was therefore possible that the eosinophils accumulated as a result of the antigen-antibody reaction or of the accompanying inflammation Experiments were therefore designed to determine whether the local eosinophilia could be produced simply by a reaction of antigen and antibody Mice were passively immunized with a commercial preparation of tetanus antitoxin and then challenged with an intraperitoneal injection of tetanus toxoid [57] The results were compared with results obtained with actively immunized animals (TABLE 1). Although a marked accumulation of eosinophils occurred in the actively immunized animals, there was no indication of an increase in the number of eosinophils in those that were passively immunized These procedures were duplicated with serum obtained from

TABLE 1
COMPARISON OF EOSINOPHIL RESPONSES IN ACTIVELY AND PASSIVELY IMMUNIZED MICE FOUR DAYS FOLLOWING AN INTRAPERITONEAL INJECTION OF TETANUS TOXOID
SIX MICE IN EACH SERIES*

	Procedure for immunization				Response to tetanus toxoid± eosinophils/cu mm peritoneal fluid (standard error)
Active	4 Subcutaneous injections of 0 05 ml toxoid during 4-week period				80660 ± 7817
Passive	A	550 units antitoxin†	1 hr prior to toxoid	‡	1208 ± 166
	B	2780	1	‡	783 ± 271
	C	550	72	‡	1117 ± 127
	D	2780	72	‡	1158 ± 417

* Reproduced by permission of *The Proceedings of the Society for Experimental Biology and Medicine* [57]

† Antitoxin—Lederle Globulin Tetanus Antitoxin (1 ml is equivalent to 5560 units)

‡ One half of the mice were injected intraperitoneally, and the remainder were injected subcutaneously

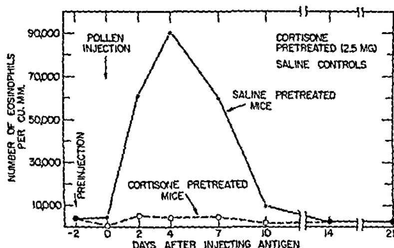


FIGURE 8 Effect of a single subcutaneous injection of 2.5 mg of cortisone acetate 48 hours prior to a challenging injection of pollen extract The eosinophil response was inhibited in the cortisone injected animals.

deal of the work that has been reported in the study of allergic inflammation or of inflammation produced by immunity reactions has involved the taking of repeated biopsies from experimental animals The failure to take precautions to avoid injury and stress probably explains why eosinophils have not been found consistently in inflammation produced by reinjection of antigens[31, 47, 64] The temporary eosinopenia that is known to occur in some parasitic and allergic diseases[2, 31] may also be due to stress or to the release of large amounts of adrenal hormones that decrease the number of circulating eosinophils. Th [illegible]

antibody[15, 24]

Another factor that can inhibit the eosinophil response is X irradiation[52] It may be seen in FIGURE 9 that a dose of 500 r to the whole body inhibits the 4-day peak eosinophil response if the irradiation is applied from 1 to 14 days prior to a challenging injection of tetanus toxoid This effect is presumably due to an inhibition of hematopoiesis and an almost complete disappearance of circulating eosinophils at the time of antigen injection On the other hand, irradiation after the challenging injection appears to result in a high concentration of eosinophils at the site of antigen injection

The effect of irradiation on the accumulation of eosinophils parallels in many respects the effect of irradiation on antibody production as reviewed by

combination of toxoid plus antitoxin was capable of producing a prolonged eosinophilia in actively, but not in passively, immunized mice.

Although Ringoen[40] noted that every animal that survived an anaphylactic reaction exhibited an eosinophilia, there is nevertheless a great deal of evidence indicating that the eosinophilia is not dependent on local or systemic shock. Schlecht[48] noted that a second injection of horse serum into guinea pigs produced both shock and a subsequent eosinophilia. However, repeated injections of serum in surviving animals produced a greater eosinophilia, but no shock.[40, 43] Samter[46] prevented the anaphylactic reactions with antihistamines and noted that the severity of the shock and the degree of eosinophilia were not related. Kark[23] also noted that the eosinophil changes take place with no relation to the manifestation or lack of manifestation of anaphylaxis.

These experiments indicate that the accumulation of eosinophils is not merely the result of shock or the reaction of antigen and circulating antibody. In the passively immunized animal local fixation of antigen occurs along with antigen-antibody reactions and a local inflammation. These responses do not produce an accumulation of eosinophils. A characteristic difference between actively and passively immunized animals is in the amount and rate of antibody production. The actively immunized animal produces new antibody very rapidly, as illustrated in FIGURE 5. On the other hand, the passively immunized animal is characterized by the slow rate of production and relatively small amount of antibody produced. The eosinophil response and the production of new antibody therefore appear to parallel one another in both the actively and passively immunized animals. This relationship is summarized in TABLE 3.

A series of experiments was performed to determine some of the factors that inhibit the eosinophil response to the reinjection of antigen.[58, 63] Mice were sensitized to a ragweed pollen extract by a series of subcutaneous injections. They were then pretreated with 2.5 mg. of cortisone acetate 2 days prior to a challenging injection of the pollen. It may be seen in FIGURE 8 that the cortisone inhibited the eosinophilia that normally follows the challenging injection. These and similar experiments indicate that an increase in the level of adrenal cortical hormones will inhibit or completely prevent the local accumulation of eosinophils at the site of antigen injection or parasitic infiltration.[10] A great

TABLE 3

COMPARISON OF THE EOSINOPHIL RESPONSE AND SERUM ANTIBODY TITER AFTER ANTIGEN INJECTIONS IN MICE

Time of challenging injection	Eosinophil response	Antibody titer
After initial injection of antigen	Slight	Low
After each repeated injection of antigen	Progressive increase	Progressive increase
Specificity	Great	Great
Time of peak response	Prior to appearance of new antibody	At the time of eosinophil disappearance
After antigen injection in passively immunized mice	Slight	Low

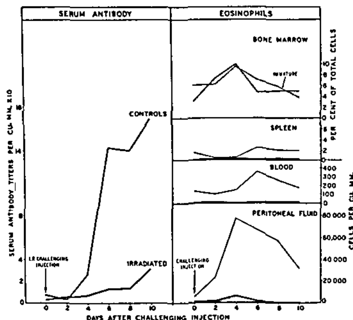
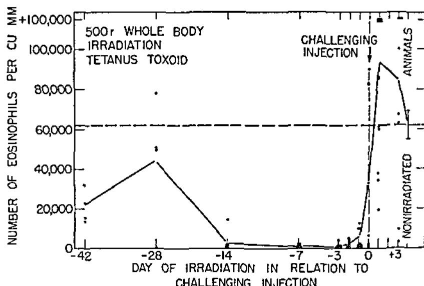


FIGURE 10 Antibody titers and eosinophil responses to a challenging intraperitoneal injection of tetanus toxoid 4 days after 500 r whole body X irradiation There was a marked inhibition of the formation of antibody and an inhibition of the eosinophil response in the peritoneal fluid

as a result of the antigen injection This increase could have been produced either by a proliferation of the bone marrow cells or by a migration of cells into the bone marrow However, the number of immature cells in the bone marrow was not sufficient to produce a significant change in the number of mature cells in the blood or in other body fluids

FIGURE 11 illustrates the effect of a challenging injection given 18 days after irradiation Note that there is a delay in the time of antibody formation and a partial recovery of the serum titer There is also a partial recovery of the eosinophil response in the peritoneal fluid Both the spleen and the bone marrow show a marked increase in the number of immature eosinophils, but the total number of segmented cells remains relatively low Thus, at 18 days there is a delayed but partial recovery of both the cellular and antibody responses to a challenging injection of antigen

FIGURE 12 illustrates the results obtained when the animals were challenged 32 days after irradiation It can be seen that there is still a delay in antibody production following the challenging injection By the tenth day the titers of the irradiated and the control mice were approximately the same On the other hand, the accumulation of eosinophils occurred rapidly in the irradiated as well as the nonirradiated animals Although the initial response was rapid, there was a marked delay in the removal of the eosinophils from the peritoneal



[illegible] *Journal of Immunology*.[53]

Taliaferro,[62, 63] Dixon,[7] Williams,[69] and others The radiosensitive stage of antitoxin formation appears to be similar or identical to the period when irradiation causes a marked reduction in the number of eosinophils responding to an antigen injection The radioresistant stage of antitoxin formation coincides with the period when the eosinophil accumulation has already begun and *will continue in spite of the irradiation Thus the effects of irradiation* on antibody formation can be explained if we assume that an accumulation of eosinophils around the antigen is necessary for antibody formation to occur.

This assumption can be tested by allowing animals to recover from the effects of irradiation and comparing the time required for recovery of the ability to form antibody with the time required for recovery of the eosinophil responses To obtain this information we irradiated immunized mice with a dose of 500 r to the whole body and gave them a challenging injection at various periods after irradiation The cellular responses were noted over a 10-day period FIGURE 10 illustrates the antibody and eosinophil response to an intraperitoneal injection of the tetanus toxoid antigen given 4 days after irradiation There was a marked inhibition of new antibody production compared with the production in nonirradiated animals There was also a marked inhibition of the eosinophil response in the peritoneal cavity and an almost complete disappearance of eosinophils from the blood and spleen It should be noted, however, that although the mature cells in the bone marrow had almost completely disappeared, *there was still an increase in the number of immature eosinophils*

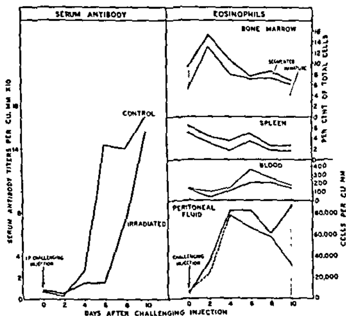


FIGURE 12.

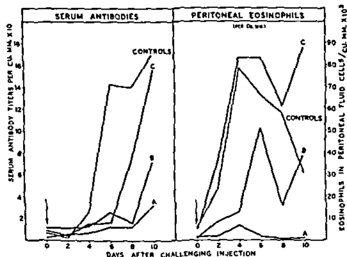


FIGURE 13.

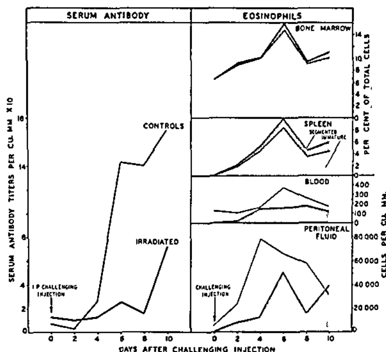
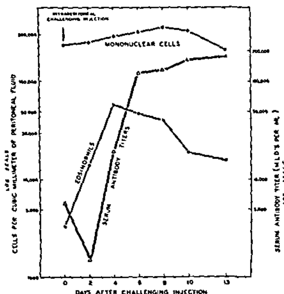


FIGURE 11 Antibody titers and eosinophil responses to a challenging intraperitoneal injection of tetanus toxoid 18 days after 500 r whole-body X irradiation A marked delay in the time of antibody formation and a partial recovery of the eosinophil response occurred

cavity. Examination of the peritoneal fluid indicated that there was a marked reduction in the number of macrophages present in the peritoneal fluid, which could account for the slow rate of phagocytosis of the eosinophils.

In FIGURE 13 a comparison is made of the rate of antibody formation and the concentration of eosinophils in the peritoneal fluid at various times after irradiation In these experiments measurements were taken of the amount of peritoneal fluid that could be aspirated into our pipettes from both control and irradiated mice In going over these data, Phelps Crump, a statistician, pointed out that there was a significant decrease in the total amount of peritoneal fluid in the irradiated animals This loss of fluid would tend to concentrate the cells in the remaining peritoneal fluid In order to take this into account, we determined the total number of eosinophils in the aspirated peritoneal fluid by multiplying the eosinophil concentration by the volume aspirated The results are plotted in FIGURE 14 It may be seen in FIGURES 13 and 14 that the antibody response closely parallels that of the eosinophil response in the peritoneal fluid This comparison is summarized in TABLE 4

FIGURE 15 compares the temporal relationship of the eosinophil and mononuclear concentration with that of serum antibody titers in immunized mice It may be seen that a logarithmic increase in eosinophil concentration occurs prior to a similar increase in serum antibody titers In all our experiments the eosinophil response preceded the appearance of antibodies in the serum



produced However, in some of our irradiation experiments (FIGURE 12) the presence of eosinophils per se in the peritoneal fluid was not immediately followed by the appearance of antibody Antibody appeared only after the eosinophils began to disappear from the peritoneal fluid owing to phagocytosis by reticuloendothelial cells

Phagocytosis of eosinophils, neutrophils, and lymphocytes has been repeatedly observed and is believed to be the primary method of eliminating worn-out or injured cells from the body In our experiments phagocytosis appeared to be the primary cause of the disappearance of eosinophils after their accumulation at the site of antigen injection Ringoen[39] noted that macrophages at the site of reinjection of antigen contain eosinophil granules, and he called these cells "special" or tissue eosinophils These granules did not appear to develop within the macrophage, but appeared suddenly, presumably by phagocytosis of leukocytic eosinophils Thus, the macrophage not only engulfs eosinophils that appear morphologically to be in good condition, but some of the components of the engulfed cells, such as the granules, may remain as distinct units within the macrophage In this manner it is possible that granulocytes, which are assumed to be end cells and to have no progeny, can pass specific enzymatic systems to cells that have longer life spans and that are capable of cell division. Plasma cells and eosinophils have been repeatedly observed in close proximity,[67] and it is interesting to note that

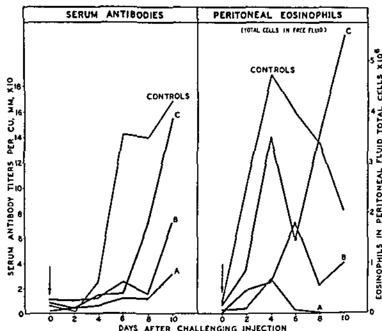


TABLE 4

COMPARISON OF THE EOSINOPHIL RESPONSE AND SERUM ANTIBODY TITER AFTER ANTIGEN INJECTION IN IRRADIATED MICE

Time of challenging injection	Eosinophil response	Antibody titer
Prior to irradiation	High	High
4 Days after irradiation	Greatly inhibited	Greatly inhibited
18 Days after irradiation	Moderate (delayed peak)	Moderate (delayed peak)
32 Days after irradiation	High (delayed removal by phagocytosis)	High (delayed peak)

In summary, our experiments, together with reports in the literature, indicate a quantitative correlation between antibody response and eosinophil response to antigen injection and suggest that one response is linked with the other. The eosinophil response precedes the formation of new antibody and appears to be essential for its production. Repeated injections of the same antigen result in a greater eosinophil response as well as a greater production of antibody. Cortisone, X rays, malnutrition, and stress all decrease the number of circulating eosinophils, thereby decreasing the number of these cells capable of responding to the antigen and resulting in a decrease in antibody

is the cell that phagocytizes the eosinophil. It is interesting to note, however, that although there has been a great deal of speculation concerning the cellular origin of antibodies, the eosinophil has not even been considered by the writers of most of the current textbooks and reviews in this field.[4, 27, 61, 70] A leading reviewer summarily dismissed both the eosinophil and neutrophil with the statement "... there is no good evidence of liberation of antibody from granulocytic cells."[70] Eosinophils certainly must be considered as part of the mechanism of immunity. This is true, not only because of the findings outlined in this report, but because of those reported in the extensive literature relating eosinophils to "detoxification" of foreign proteins in allergic diseases, chronic inflammation, and most infective diseases characterized by immunity reactions.[1-3, 6, 16, 22, 33, 40, 44, 48, 49, 51, 56, 64] Moreover, there are a number of papers in the literature suggesting that eosinophils are involved in the production of antibodies.[16, 19, 49, 52, 54, 57, 66]

In view, therefore, of the observations presented in this report and those recorded in the literature,[4, 7, 12, 13, 27, 30, 41, 60, 61] and with some deference to the reutilization hypothesis of Hamilton and Trowell and to the lymphoid origin of stem cells proposed by Yoffey (which have been presented in detail elsewhere in this monograph), I propose the following hypothesis.

Antibody production is first initiated by the introduction of antigen into the tissue fluids somewhere near a blood supply. The presence of this material produces an inflammatory response in which both neutrophils and eosinophils migrate out of the blood vessels and into the surrounding area. The role of the neutrophil appears to be obvious, it acts as a phagocyte and releases proteolytic enzymes that assist in the breakdown of the antigenic material. The role of the eosinophil is not so obvious. It is chemotactically attracted toward the antigen but, unlike the neutrophil and macrophage, it does not actively phagocytize large quantities of antigen nor release proteolytic enzymes. Instead, chemical changes occur in or on the surface of the eosinophil, forming what the immunologists refer to as an enzymatic template. The eosinophil is then phagocytized by a reticuloendothelial cell that is in the vicinity of the antigen or in the draining lymph nodes and spleen. This macrophage then undergoes a change in its morphological appearance and in its physiological function. The cytoplasm becomes more basophilic, and the cell ceases to act as a macrophage. It begins to synthesize antibody, utilizing the enzymatic template formed by the eosinophil. After a short period of active antibody production the reticuloendothelial cell is carried through the lymph and blood vessels and eventually makes its way to the bone marrow. In the bone marrow, it undergoes mitotic division, forming immature and, finally, mature eosinophils. These new eosinophils have within them certain enzymatic constituents that were transmitted to them from the original eosinophil that had first reacted with the antigen. The new eosinophils therefore have a greater affinity for the specific antigen, and a greater number of them react to each successive injection of the antigen. The increase in the number of these cells containing the preformed enzymes from the parent reticular cell results in greater and more rapid formation of new antibody.

The above hypothesis indicates that cellular reaction and antibody formation

Moeschlin[30] observed granular material in plasma cells at the time that eosinophils were being phagocytized and antibody was being produced. It is quite possible that these granules are remnants of phagocytized eosinophil granules

It is generally agreed that antibody production occurs in the lymphoid macrophage system,[61] in which reticuloendothelial cells, lymphocytes, plasma cells, transitional cells, and eosinophils exist side by side[28] Similar cells are found in the inflammatory exudate that has been produced by repeated injections of antigenic material.[41, 65] The cells in this exudate, as well as those in the lymphoid tissues, have been shown to be capable of producing more antibody when transplanted into animals not normally capable of antibody production[60] Practically all the theories of antibody formation involve one or more of these cells

At the present time we have no data suggesting that eosinophils per se produce antibody In a preliminary experiment we have attempted to assay peritoneal exudate containing large numbers of eosinophils The antibody titers generally have been low, and they show no relation to the number of eosinophils in the cellular population. On the other hand, from our data and from the extensive data in the literature, we have reason to believe that the cells that have phagocytized the eosinophil contain antibody and are capable of producing more antibody if transferred to an *in vitro* situation Experiments by Kolough *et al*,[26] Fagraeus,[12] Roberts *et al*,[41] and others have indicated that during the time antibody is being synthesized the macrophages increase their cytoplasmic basophilia and then develop into plasma cells Numerous other experiments, however, have indicated that these phagocytic cells are not capable of initiating antibody production if the antigen is added directly to the cells *in vitro*[9, 14, 25, 36, 45, 50] On the contrary, the reaction of phagocytic cells with the antigen leads to digestion of the material and a decrease in the resulting production of antibody[42, 60, 65] An *in vivo* reaction appears to be necessary before the *in vitro* production of antibody will occur.[36] This suggests that an inflammatory response is the missing prerequisite for antibody production In other words, the cells that eventually produce antibody must remain *in vivo* for a period of sufficient duration for hematogenous cells to migrate out of the blood vessels and react with the antigen Thus, antibody production appears to be initiated by blood leukocytes, but completed by macrophages during the period of their transformation into plasma cells

Experimental data presented here and by numerous other workers[4, 7, 20, 63] suggest that two of the stages involved in the production of antibody are

(1) A stage consisting of the reaction of certain cells with antigen, producing specific enzymatic changes or forming an enzymatic template This stage occurs *in vivo* at the site of penetration of the antigen into the organism It is inhibited by irradiation

(2) A later stage, consisting of the utilization of specific enzymes for the synthesis of antibody This stage can occur *in vitro* as well as *in vivo*, it takes place primarily in the lymphoid or the reticuloendothelial tissues It appears to be less affected by irradiation

It seems highly probable to us that the cell involved in the first stage of antibody production is the eosinophil and the cell involved in the second stage

are not only cyclic, as shown in FIGURE 16, but spiral as well Each time the animal goes through the cycle there is an increase in the magnitude of eosinophil response, an increase in the number of eosinophils phagocytized by reticuloendothelial cells, an increase in the amount of resulting antibody and, finally, an increase in the production of new eosinophils This hypothesis should at least stimulate workers in the field of immunity to think in terms of the fact that the formation of antibody may involve more than one cell. Antibody production definitely occurs in stepwise fashion, and it seems very probable that the first step involves an eosinophil The hypothesis can easily be tested, especially by those who work with tissue cultures *In vitro* production of [illegible]

[illegible] lial cells.

Possibly of greater importance is the concept of a cyclic system of closely [illegible] in [illegible] by [illegible] the engulfed cellular components to carry out specific physiological functions, after which they divide to produce new myeloid cells The reutilization of [illegible]

Acknowledgments

I express my thanks to the following persons, each of whom has cooperated in certain aspects of these experiments

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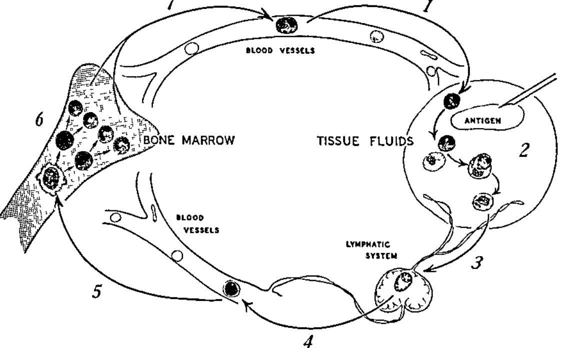


FIGURE 16 Cyclic pattern of eosinophil responses to antigen

(1) Chemotactic response of blood eosinophils to the antigen. There is a reaction between the eosinophil and the antigen, forming a hypothetical enzymatic template.

(2) After reacting with the antigen, the eosinophil is phagocytized by macrophages. These reticuloendothelial (RE) cells then increase in cytoplasmic basophilia and begin to produce antibody.

(3) The macrophage loses its ameboid motility and is carried into the lymphatic system. It can be found in large numbers in the draining lymph nodes and, later, in the spleen.

(4) After antibody production has ceased, the cell, now resembling a lymphocyte, is carried via the thoracic duct to the blood stream.

(5) The RE cell eventually finds its way into the bone marrow, where it enlarges to form a stem cell.

(6) The stem cell divides by mitotic division, forming immature and, finally, mature eosinophil cells.

(7) The eosinophils are passed out of the bone marrow into the blood vessels, where they can repeat the cycle. Each time this cycle is repeated there is an increase in the number of eosinophils produced, and a corresponding increase in the number capable of responding to the antigen.

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EVIDENCE FOR THE VIRUS ETIOLOGY OF HODGKIN'S DISEASE*

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Among human tumors, Hodgkin's disease has long held a peculiar position, not only because of its clinical manifestations in the patient, but also because of its appearance under the microscope As well as progressing in its victim as a uniformly fatal disease of a tumorous type that predominantly involves lymph nodes, it often manifests itself with striking symptoms of toxicity This latter characteristic reveals itself as recurrent intermittent episodes of fever, malaise, night sweat, enlarged nodes, and debility. These Pel-Ebstein cycles are strongly reminiscent clinically of an infectious type of reaction This impression is reinforced by the microscopic pattern of a pleocellular type of reticulum and connective tissue cells intermixed with eosinophils, granulocytes, plasma cells, Sternberg-Reed cells and, sometimes, necrosis There is a peculiar lack of microscopic evidence of the relentless proliferation of basically a single type of malignant cell that is the customary picture in the usual malignant tumor.

Because of these provocative properties, Hodgkin's disease has repeatedly been the object of tumor research directed toward finding a specific etiological agent Comprehensive reviews of the literature on this disease[1-4] have demonstrated repeatedly that this tumor seems to be halfway between inflammation and neoplasia During the golden era of bacteriology it was only natural that extensive research should be directed toward an analysis of the possible role of bacteria in Hodgkin's disease. Sternberg,[5] in 1898, originally considered that tuberculosis was of causative significance, and this possibility was entertained as recently as 1931 by L'Esperance,[6] although the work of Stewart[7] and others has effectively demonstrated that such infection is not of primary significance

Other bacteria have been carefully scrutinized, especially the diphtheroids by Bunting and Yates[8] and *Brucella* by Forbus *et al*,[9] only later to be discarded as evidence was presented that these organisms were not the cause of the disease and were sometimes found in both normal and neoplastic lymph nodes Fungus infections have been related to Hodgkin's disease in much the same manner[10] With the recent increased use of antibiotics, more and more instances of the association of tumors, lymphoma, and Hodgkin's disease with fungus infections are being reported[11]

Careful research has so repeatedly failed to demonstrate a relationship of Hodgkin's disease to bacteria and fungi that investigations from that point of view have been essentially abandoned The only remnants of a significant possible relationship between Hodgkin's disease and large microorganisms (bacteria and fungi) are to be found in the concept that Hodgkin's disease is

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sessing the size and shape of known viruses was confirmed In avian lymphomatosis, especially, and in erythromyeloblastosis to a lesser extent, the tumors were recognized as occurring sporadically in flocks of birds, but there is little evidence to support the possibility of an infectious spread. Even inoculations were sporadic and unpredictable in their effect, many animals apparently being resistant. The first major evidence for virus etiology was the demonstration that filtered extracts would occasionally cause the tumors The previously known successful filtrability of the Rous sarcoma material oriented the thinking in regard to the lymphomatosis group The effect and existence of immune bodies were demonstrated; consequently, by the time that small viruslike particles were seen, the groundwork was laid for the general acceptance of the virus cause of the disease.

In the mouse leukemia of Gross, the original tumors had seemed noninfectious and appeared to develop sporadically in the colony It was the discovery of the importance of age in regard to susceptibility to transmission that permitted the demonstration of an infectious agent. Filtrability supported the contention of a virus factor, yet even now the examination of the tissue for virus reveals particles that are morphologically suggestive, yet whose identity and significance have not been clearly established

In reviewing these "well-accepted" virus-caused malignancies, it is apparent that the basis for their acceptance is not the fact that they have fulfilled Koch's postulates Instead, the virus etiology has been indicated by a series of experiments that suggest a high degree of probability for virus causation What comprises the criteria that are generally accepted? The ability to pass through filters that are demonstrated to keep out bacteria-sized particles is particularly important, this property is considered to relate the agent to the virus magnitude of size Second, evidence is amassed to demonstrate that the agent multiplies, and is thus accepted as living Usually this is achieved by showing that after a series of animal or tissue passes, the agent retains its full activity, thus demonstrating that reproduction must have occurred The third and final evidence requires that this filtrable and reproducing agent show that it produces an increased incidence of the tumor under study in its host These are the three basic generally accepted criteria

It is to be noted that the "virus" referred to need never be seen or, at least, the evidence is not much more acceptable if viruslike particles can be demonstrated The production of a tumor in another species of susceptible animal is not done because of the high specificity at this type of agent It is seldom that the agent can be inoculated "in other susceptible animals" of a different species with any resultant tumor formation Also, the agent need not be found only "in the body in accordance with the lesion observed," since it is often in tissues *other than the tumors proper and is sometimes demonstrable* in animals showing no tumors [20] By the use of tissue cultures it is usually possible to "cultivate the organism outside of the body "

Virus-Caused Tumors in Man

The opportunity for studying benign tumors, especially epithelial, in man is great, however, because of their relatively little clinical and medical signifi-

a malignant degeneration of a lymph node that may occur following any one of a series of chronic irritations in such nodes. In this concept, such factors as bacteria, fungi, viruses, and allergies are visualized as repeatedly irritating a lymph node with resultant hyperplasia which, if prolonged and vigorous enough, may turn into a malignant process[12] This has recognizable parallelism with the concept of the malignant and carcinomatous degeneration that follows irritated and inflamed lesions, such as those of the cervix uteri and the unhealing sore of the lip As the primary causative significance of bacteria and fungi has become less probable it is only natural that attention should be directed toward viruses as a possible cause. Gordon,[13] after observing rabbit encephalitis consequent to intracerebral inoculation of Hodgkin's disease lymph node material, was the first research worker to undertake an investigation in this field By that time the significance of viruses in some animal tumors was known, so that several research workers soon embarked upon projects with that possibility in mind. Much of this work has been reviewed by Bostick.[14]

Generally Accepted Evidence for Virus-Caused Tumors

There is a general tendency to assume that Koch's postulates must have been fulfilled before accepting a virus as the cause of a given tumor, since this constitutes such a basic tenet in bacterial diseases It will be worth reviewing selected tumors to see to what degree this requirement has been achieved These postulates involve. (1) demonstrating the organism in all cases of the disease and showing that it is distributed in the body in accordance with the lesions observed, (2) cultivating the organism outside of the body; and (3) using the isolated organism to reproduce the disease in other susceptible animals It is clear that, in general, these requirements have been most difficult to fulfill in virus diseases The organisms are consistently so small and their morphology is so nondistinctive that one is unable to demonstrate their presence *in the tissue or differentiate them with certainty from other bodies of the same* order of size Sometimes the stumbling block is the third postulate, in that there is an inability to find another animal that will reproduce the disease in question, although viruslike particles may be demonstrated repeatedly in the lesions of a particular disease in the original host

Only in a very few diseases of plants have the postulates actually been fulfilled in all details for the virus disease, thus, in the vast majority of cases, complete proof is lacking, and the scientist is forced to rely upon variable degrees of probability as to the causative role of a particular virus in a disease

There are several malignancies in animals that are quite generally accepted as being of viral origin These include the mammary tumor of the mouse,[15] the Rous sarcoma of the chicken,[16] avian lymphomatosis,[17] and erythroblastosis,[18] and certain types of mouse leukemia[19] The variety of types of evidence that have been accepted in establishing their viral connection is interesting

At one time the mouse mammary tumor was considered as possibly being caused by a sex-linked hereditary factor Later, its existence in many tissues and its filtrability were established and, finally, the presence of an entity pos-

The principal human tissue to be employed will of necessity be the tissue culture Since, in a tissue culture, neoplasia cannot be identified and does not possess any specific structural characteristics, other evidence for the action of

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sought,

since viruses that are neoplastic in some circumstances may incite inflammation in others.

In human tumors any "factor" would have to produce some lesion, neoplastic or otherwise, that was demonstrable after serial passages, since the ability to reproduce is an essential criterion of a virus In this regard, the evidence is similar to that used in animal virus tumor work Comparable also is the need to demonstrate that the factor is in the size range of viruses This is most easily shown by filtration methods, electron microscopy, or differential centrifugation Finally, in man, much emphasis necessarily must be placed on various serologic and immunologic procedures, and this is by no means easy However, it is one of the basic ways by which to relate the factor to man and to be certain that it has not been isolated from animals used in experiments involving such media as tissue culture, mice, or fertile eggs After relating it to man by demonstrating antibodies to it by such means as neutralizing and complement fixing, the factor must finally be correlated with the neoplastic state by more refined, specific, and special serologic procedures The ultimate proof would lie in the proper selection by elaborate means of the susceptible human volunteer, together with enough time for any lesion to develop

The experimentalist in this field always hopes that the final proof will come more easily An especially happy situation would be the development of an antibody to the supposed human cancer agent that could be used therapeutically to cure, to prevent, or to deter the spread of the cancer This circumvention is most unlikely if one can use the animal virus tumors as guides Effective vaccines will prevent the onset of the specific cancer and will immunize against the cancer virus, but will not prevent the growth or spread of the cancer once it has started As with many virus diseases, the agent seems to be beyond the reach of ordinary humoral antibodies once it is actively and securely lodged in the carcinogenic cells that are its target

tumors much basic research must be done, and even greater effort is needed in the virus approach to malignant tumors and Hodgkin's disease This is not to deny the significance of the results obtained, but to emphasize the greater magnitude of the problem when the process under study is malignant

Evidence of a Virus in Hodgkin's Disease

Hodgkin's disease has been the object of numerous experiments designed to detect possible infectious and virus agents Many of the experiments have been of a preliminary type or composed in such a manner as not to permit

cance and importance, few comprehensive experiments have been reported. That is unfortunate because of the great theoretical importance of basic data of this kind in evaluating the relationship of tumors in man in general Clearly, in the instance of benign tumors, human inoculations can be used and Koch's postulates approached without the fear of dangerous complications that would arise in the studies with malignant tumors.

Perhaps the single benign tumorous proliferation in man that has been best demonstrated as being of virus origin is the simple epithelial proliferative lesion, molluscum contagiosum. With inoculated human volunteers the incubation period varies from two to seven weeks. The agent is filtrable and

size. Man is the only known host, since repeated attempts to infect other animals have not been successful; thus it is apparent that the postulates have not been fulfilled in this regard

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68 *mμ* in diameter in cutaneous papillomas. Whether the prominent "inclusion bodies" so frequently encountered in rectal papillomas are evidence of their virus nature[24] has not been clarified This applies also to the status of the viruslike bodies encountered by Meesen and Schulz[25] in laryngeal papillomas.

Although one could add other examples to the list of benign human tumors of possible virus origin, it suffices for this discussion to note that the evidence *for a virus cause is always incomplete Even so, the acceptance may be quite* general in spite of the lack of positive identification of a virus Again, as in animal tumors, factors of filtrability and serial passage are of great significance Actual demonstration of a virus particle, although interesting, is of less importance

Malignant virus-caused tumors in man have not been demonstrated conclusively Seldom has it been possible to pursue the avenues that have been *shown to be fruitful in animal virus malignancies Moral and ethical rules,* of course, stand in the way and limit the numbers and types of experiments that would be necessary A few inoculations would not constitute a true test, be-

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malignant virus tumors in man to different avenues, to approaches that do not involve the natural host directly In this approach the weight of evidence must temporarily shift somewhat The presence of a virus particle demonstrably visible becomes more significant Evidence of suggestive virus action in cells, such as inclusion bodies and elementary bodies, must be sought

disease research, it is apparent that emphasis must be placed on the use of human cell line tissue cultures. The methodical use of numerous strains of such cultures in studying each Hodgkin's disease tissue should provide a significant avenue of research in any laboratory interested in etiological Hodgkin's disease studies. As will be discussed under the properties of the virus isolated from mice inoculated with Hodgkin's disease, it has been possible to pass that virus through three serial cultures of monkey kidney cells without causing any cytopathogenicity in them

Animal inoculations *Practically no species of laboratory animal has escaped* inoculation with some form or type of Hodgkin's disease material Monkeys,[4, 33, 34] rabbits, mice, guinea pigs, rats,[3, 35] pigs, chickens, pigeons, and dogs[36] have been included The inoculated material has comprised Hodgkin's disease ground tumor, fresh fragments supernate, tissue culture, and tissue culture supernate. These materials have been administered in erratic but numerous routes such as: intramuscular, subcutaneous, intraperitoneal, intravenous, and intracerebral, into the spleen, lymph nodes, and liver, and also into the respiratory and gastrointestinal tracts In no instance has a recognizable disease that suggested a neoplastic process developed

The encephalitis produced in rabbits upon intracerebral inoculation is well known and was first discovered by Gordon *et al*[13] Although this is most interesting, later study failed to demonstrate that it was serially passable or a viral agent[37-39] Guinea pigs have been reported occasionally to develop a small distinct nodule upon subcutaneous injection of ground Hodgkin's disease tissue.[13, 35]

The young and also embryonic stages of animals have been studied in their reactions to Hodgkin's disease tissues, and no neoplastic reaction has been reported in them Both Karnofsky *et al*[40] and ourselves have noted edematous

embryonated chicken eggs The eggs were inoculated after seven days of incubation, and the mortality was compared ten days later with parallel series of non-Hodgkin's disease tissue-inoculated control eggs Although slight, the mortality was greater in the Hodgkin's disease eggs and, when comparing large groups of eggs, became statistically very significant

Some investigators have reported the use of intra-uterine and newborn animals We have done intra-uterine inoculations of guinea pigs without causing demonstrable disease in the mother or offspring We have also inoculated pigs when young adults, as well as when newborn, with and without serial passage of the material, without demonstrable effect The inoculation of newborn Princeton mice intracerebrally with original Hodgkin's disease extract, as well as with serially passed amniotic fluid from Hodgkin's disease-inoculated eggs, has been studied[42, 44] with special thoroughness by investigators who injected newborn mice less than 24 hours old, intracerebrally, with the material to be tested On the tenth day the mouse brains were harvested, ground, and inoculated intracerebrally into the next litter of newborn mice

objective analysis Others have been carefully done, but with completely negative results. It is not the intent of this presentation to review all the experimental literature in Hodgkin's disease, since this has been done elsewhere.[4, 14, 26] Instead, results and papers that present evidence supporting *the theory of an agent in Hodgkin's disease will be noted and discussed.* If data that cast doubt on such supportive evidence are available, they also will be presented

Tissue culture techniques. Both culturing of actual Hodgkin's disease tissue and the inoculation of Hodgkin's disease material into tissue cultures of other non-Hodgkin's disease tissues have been the methods used Studies of cultures of original Hodgkin's disease tissue have been done frequently.[27-29] Their interpretation is difficult, since there is a great technical variability in such explants, including size, fibrous tissue, necrosis, and serum- and blood-transferred viability. This has permitted a large subjective factor in reported effects, and has been recognized by most authors Liquefaction of the substrate was often prominent, as were fat vacuoles, increased free cell forms, and more frequent multinucleated cells. These effects do exist, and they probably occur more in Hodgkin's disease tissue than in any other; however, it is the general feeling that there is such great individual variation in the various cultures, and thus in their interpretation, that there is little practical importance to the observation

In observing the effects of Hodgkin's disease material such as serum and tissue extracts on organoid tissue cultures (including lymph nodes and liver) of both human and animal origin, the technical obstacles have been similar to those described above in studying the growing Hodgkin's disease lymph node material itself Here, too, the effect of Hodgkin's disease material on human tissue, chick embryos, and mouse lymph node seems to result in abnormal tissue cultures.[28, 29] Because of the true and subjective variability of these preparations *and observations, it is apparent that only by the use of pure* cell line tissue cultures can the effects of Hodgkin's disease extracts be fairly and objectively evaluated

Studies on HeLa cells in our laboratory have failed to detect any cytopathogenic effect of extracts of either Hodgkin's disease lymph nodes or of the supernatant fluid removed from cultures of Hodgkin's disease node explants. Using a Genetron* extraction of Hodgkin's disease lymph nodes (see under *Viruslike particles in Hodgkin's disease extracts and passages* below), some cytopathogenicity on HeLa cells has been noticed Limited similar *inoculations into* culture of Chang's[30] liver cells and Bell and Johnson's[31] lymph node cells have been unrewarding. The effects of the supernate of Hodgkin's disease tissue culture explants were tested because of the possibility that such fluid might contain a virus agent that had been released from the original Hodgkin's disease tissues Similar experiments were carried out, using human amnion sac cells,[32] as well as monkey kidney cell cultures, without observed significant effects

Because of the great difficulties of employing human subjects in Hodgkin's

* Genetron, Purple Label 113 (trifluorotrichloroethane), produced by the General Chemical Division of Allied Chemical and Dye Corporation, New York, N Y

colonies used in our experiments Theiler's G D VII antiserum failed to neutralize our virus Antiserum to our virus did possess the ability slightly to reduce the titer of both known G D. VII and FA virus strains. Of significance in the Hodgkin's disease-isolated virus is the fact that, although newborn mice are susceptible, suckling mice are much more resistant, and weanling mice are most difficult to infect No immune neutralizing antibodies to the Hodgkin's disease-isolated virus have been detected in the serum of adult mice from our colonies

Gordon[49] encountered "elementary bodies" in Hodgkin's disease material. He tested the sera of 18 patients with Hodgkin's disease with several preparations of his antigens and reported only variable results Grand[50] prepared a purified suspension of human Hodgkin's disease lymph nodes and immunized rabbits with it He later tested the rabbit serum for the presence of agglutinins for Hodgkin's disease node extract and concluded that agglutination of very small particles was noted in Hodgkin's disease material An exhaustive complement fixation method study of Hodgkin's disease serum, using complement fixation tests and collodion particle agglutination was done by M S Hoster.[4] The tests were negative

Although hemagglutination (HA) has been searched for in Hodgkin's disease extracts, sera, and tissues,[4, 43] it has not been present nor different from control tissues Hemagglutination has become of significance in this research only when used as a tool to detect the growth of known hemagglutinative viruses Bostick and Hanna[43] made a systemic study of the hemagglutinative capacity of the amniotic fluid from chick embryos that had been inoculated with Hodgkin's disease lymph node extracts Such amniotic fluid showed no HA power. The experiments were then modified by using viruses that are known to be hemagglutinative and by inoculating them into Hodgkin's disease-injected eggs in order to try to demonstrate a possible interference phenomenon Specifically, 5- to 7-day incubated fertile eggs were first inoculated with various Hodgkin's disease extracts or serially passaged Hodgkin's disease material After 3 days these same eggs were challenged with an inoculation of influenza Lee virus Eighteen hours later, the harvested amniotic fluid (AF) was tested for the amount of influenza growth by its hemagglutination titer[51]

These experiments, which are a variety of virus growth interference studies, were done on a large series of Hodgkin's disease-inoculated eggs[52] On many occasions it was possible to demonstrate an interference phenomenon between the inoculation of Hodgkin's disease material in eggs and the growth of Lee virus Giordano[53] observed similar reactions in other experiments of this type Of all the interference tests run on Hodgkin's disease-inoculated chicken AF, 60 per cent showed interference phenomenon, 27 per cent showed no interference, and 13 per cent of the tests showed variable degrees of reversal

The capacity of Hodgkin's disease-inoculated amniotic fluid to interfere with the growth of the Lee virus to a greater extent than non-Hodgkin's disease material inoculations does not establish a virus factor However, when it is recalled that the Hodgkin's disease material can be serially passaged from amniotic sac to amniotic sac and still seem to retain this effect, the ob-

Twelve different extracts from different Hodgkin's disease patients were so studied, along with parallel control material. On 3 separate occasions, after from 6 to 10 blind serial passes, the Hodgkin's disease group of mice developed a fatal encephalitis Two lines of viruses were permanently established The disease they caused is fatal to the infant mice in 7 to 10 days. The virus agent could be inactivated at 56° C in 30 min. and was Seitz filtrable and ether resistant. Muscle homogenate would not pass the disease After repeated attempts it was possible to adapt to passage by intraperitoneal inoculation

Microscopically, the moribund mice presented an encephalitis reaction that was nonspecific in pattern. There was no evidence of a neoplastic or granuloma reaction, either in the brain tissue or in the spleen, thymus, or lymph nodes in the case of the reticuloendothelial-peritoneal-adapted virus Examination of the purified material from the harvested cerebral tissues of the Hodgkin's disease-inoculated mice revealed spherical particles 32 mμ in diameter that are not in the control inoculated mice.[44] Certain serologic properties of this virus will be presented below under *Serologic method.*

It is apparent that the isolated virus, even though encountered on three separate occasions, has not been positively shown to come from the material that was inoculated into the mice and therefore may not be from Hodgkin's disease. Although the demonstrated agent may be an activated latent virus originally present in the experimental mice or even from the chicken eggs, in one instance this thesis has not been proved. Especially important, it has not been encountered in the control injected series of mice It may be a human Orphan virus Certainly, its significance is in doubt, and further studies must be and are being made

Serologic method This is a most difficult approach to Hodgkin's disease, since these patients characteristically fail to react to antigenic stimuli in a normal manner[45, 46] This relative energy decreases the probability of our being able to demonstrate circulating antibodies and serologic reactions to suspected etiological agents being tested There are no actual tests that can

the aspect of possible virus

to test It is true that in

may occur with increased

frequency[47,48] However, their significance is obscure

So far, the only viruslike agent that has been available for testing has been the virus isolated from the Hodgkin's disease-inoculated mice[43, 44] This virus was not neutralized when tested against serum from 7 patients with Hodgkin's disease, nor against 7 normal human sera. Pooled human gammaglobulin, when diluted 1:10 and treated as a routine serum, did show neutralizing activities, however, individual Hodgkin's disease and normal human sera did not, nor did they possess complement fixing antibodies against the virus

regard

It is much more difficult to rule out the Theiler's group of mouse encephalomyelitis It is latent in many colonies, although not known to occur in the

the reticuloendothelial (RE) system by Weiss. Jacquez and Porter[56] examined Hodgkin's disease material from tissue culture at 17,000 magnification and noted no structures resembling virus particles. Hoster *et al*[57] examined extracts of Hodgkin's disease tissue for particles by EM along with their studies of macromolecular particles They did not encounter any uniformly sized particles that appeared specific. H M. Richter[58] describes some large particles isolated from Hodgkin's disease material. His pictures are mediocre; the particles are approximately 80 mμ in diameter, and their characteristics are inadequately described André *et al*[59] described the EM of some cells from Hodgkin's disease lymph node punctures They record the morphologic details of the cell, but do not comment upon any virus-type structures

In our EM survey of Hodgkin's disease tissues the specimens have been fixed in osmic acid within 3 minutes after their surgical removal by the method of Caufield[60] After sectioning to a thickness of 1/20 mμ the embedded tissues are examined for evidence of structural changes, with special reference to possible inclusionlike bodies or viruslike particles. The great majority of cells studied showed no significant structures that attracted attention Occasionally we encountered cells that had inclusionlike bodies (FIGURES 1-6) Magnification at from 30,000 to 44,000 demonstrated very small particles in and around such bodies that appeared solid or, on cross-section, doughnut-shaped

A general review of EM findings in sections of tissues has been presented by Selby[61] Dalton and Felix[62] present a further discussion of this topic with electron photomicrographs of very small particles in cells from mouse malignancies, and Dmochowski and Grey[20] and Graffi[63] compile data and micrographs of tissue sections from mouse mammary tumors and leukemia tissues Palade[64] discusses small particulate matter in electron micrographs in general Clearly, the demonstration in such preparations of inclusion and viruslike bodies is suggestive, but does not prove their identity or significance Exhaustive research with purification would be needed to advance in that direction to the degree of certainty. Nevertheless, such bodies are additive data that, when assembled with other evidence of the presence of a virus, gain increasing significance

Viruslike particles in Hodgkin's disease extracts and passages Two types of material have been examined for viruslike particles in our Hodgkin's disease studies One has comprised the brain tissues of mice inoculated with serially passed Hodgkin's disease lymph node material These serial passage experiments have been described briefly under *Animal inoculation* above and in detail by Bostick and Hanna[41] The second type of material has been actual ground Hodgkin's disease lymph nodes The mouse brain homogenate material was purified and concentrated by use of a modification of the butanol extraction method for virus purification in tissues developed by Bachrach and Schwerdt[65]

The mouse brain was homogenized and made into a 10 per cent suspension in 0 1 M NaCl adjusted to *p*H 4 5 with 2 M acetic acid The precipitate was resuspended in a veronal acetate buffer of *p*H 9 0 plus 1 volume of cold *N*-butanol and emulsified for 0 5 hours in a Waring Blendor All procedures are carried out at 4° C. The resultant emulsion was centrifuged at 4000 rpm

servation becomes more pertinent. Presumably, whatever agent causes this effect is filtrable and serially passable This always conjures up the visions of a possible virus agent. Beyond that it is not yet possible to go

Hodgkin's disease cell structures and viral particles The often characteristic effects that viruses have on cells are well known. The production of necrosis, lysis, mitotic stimulation, elementary bodies, and inclusion bodies are some of the more important responses. Although rarely specific, certain of the changes are highly suggestive of a virus-type response, so that intracellular structure should be examined in any search for a virus etiology of a given lesion

Although degenerative lysis of certain cells (as of the anterior horn cell in poliomyelitis) and granulomatous inflammation (as in lymphogranuloma venereum) are very suggestive in these types of infections, it is usually the intracellular bodies that are most informative. Both "elementary" and "inclusion" bodies occur in virus infections There is little need to distinguish between them, although the former may be more basic and may represent actual individual virus particles or small clumps of them. Perhaps the latter is more frequently thought of as denatured or condensed masses of protein occurring with the virus-injured cell.

In ordinary light microscopy these structures have no specific appearance They may stain either basophilic or acidophilic, are well circumscribed, are usually in characteristic position in the cell, and possess no fine detail of morphology They are easily confused with artifacts such as protein precipitate, stain masses, and cell fragments With electron microscopy, virus masses and elementary bodies are seen to have often a rather characteristic appearance This will be discussed later

In Hodgkin's disease, structures morphologically acceptable as elementary and inclusion bodies by light microscopy are present From the very first speculations of a virus relationship, the large dusky rose-colored, so-called nucleolus of the Sternberg-Reed (S-R) cell has been considered as being a possible inclusion body. Gordon[13] suggested that the small particles demonstrable in photomicrographs from Hodgkin's disease tissue extracts are elementary bodies Grand[29, 54] was impressed by the large irregular "inclusions" stainable by brilliant cresyl blue and present in Hodgkin's disease reticulum cells and S-R cells. Rottino[27] observed the same type of bodies, but he regarded them as nonspecific and present in control material Hoster *et al* [55] used a ten-step differential ultracentrifugation in order to separate out various components of macromolecular particles from Hodgkin's disease, lymphosarcoma, and normal tissues They noted a prominent particle size in the 10- to 20-mμ range in Hodgkin's disease material

It is clear that Hodgkin's disease possesses sufficient inclusion and elementary-type structures to warrant a high degree of suspicion with regard to their possible virus significance However, a definitive or even a strongly suggestive opinion cannot be ventured from light microscopy alone Further analysis is necessary at the electron microscopy level

Electron microscopy Quite a few workers have now studied ultrathin sections of *Hodgkin's disease tissues under the electron microscope* (EM) Elsewhere in this monograph a paper is presented by Frajola, also one on



FIGURE 2 The "inclusion body" at higher magnification is not lined by a nuclear t membrane, and consequently is not a nuclear infolding Very small particulate bodies seen, some of them having a doughnut configuration × 44,000



FIGURE 1 Cytoplasmic "inclusion body" (I) in reticuloendothelial type cell present in a Hodgkin's disease lymph node Electron photomicrograph × 19,500

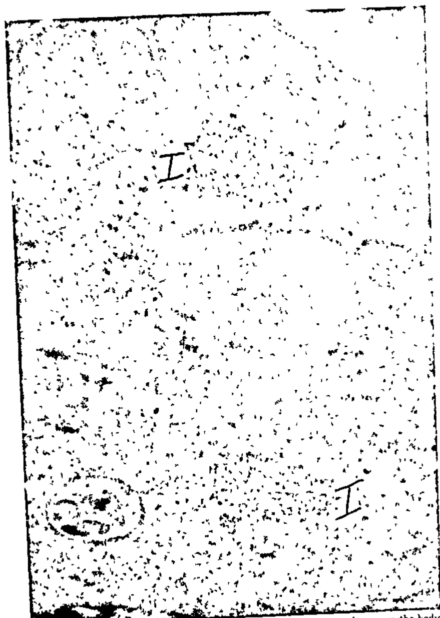


FIGURE 6 The particles with the holes in the centers are more easily seen in the bodies (I) that lie in the lighter portions of the electron photomicrograph × 30,000



FIGURE 5. Electron photomicrograph of a large reticuloendothelial-type cell from a Hodgkin's disease lymph node. A well circumscribed nuclear infolding (NI) is apparent. Besides the irregular nucleolus (NcL) several islands of "inclusion-body" type, composed of particulate matter, are visible. × 11,440

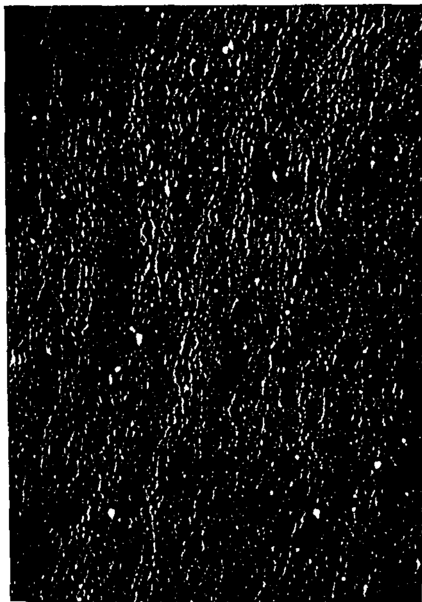


FIGURE 7. Shadowed electron micrograph of purified material from experimental serially passaged Hodgkin's disease injected mouse brain. Note the spherical viruslike bodies 31 mμ in diameter, found only in the experimental groups and not in the controls. × 56,000

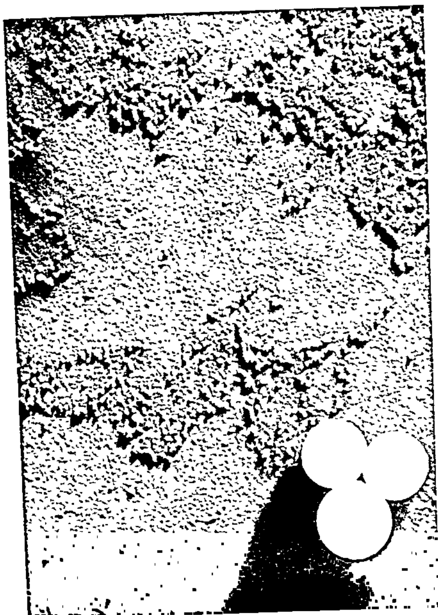
for one half hour. Of the 4 distinct phases formed, the aqueous was saved, the gel, butanol, and pellet were discarded The aqueous phase was then emulsified again for 0 5 hours with butanol, as already described. The aqueous phase was saved, and centrifuged for 2 hours at 78,000 g The resultant pellet was resuspended in 0.1 M NaCl and, after a final centrifugation at 9000 g for 10 minutes for clarification, was retained for electron microscopic examination as a shadowed preparation.

By this method it has been possible to demonstrate viruslike particles in a highly purified and concentrated form These particles measure approximately 30 mμ in diameter and were not present in control mouse brain material (FIGURES 7 and 8). As is apparent from the report in the literature,[43] the relationship between these viruslike particles found in the mouse brain and Hodgkin's disease is not proved The mice had been inoculated intracerebrally when newborn and, after 10 days, their harvested brains had been passed into new litters of newborn mice This was continued until ultimately the animals, after about 8 serial passages, developed a fatal encephalitis The ground-up brains of these sick animals contained the viruslike particles The fact remains, however, that the *particles were present and were morphologically* quite typical These experiments gave the impetus for studying by related methods homogenates of original Hodgkin's disease material

The method adopted for the purification and study of Hodgkin's disease tissue and fluid was one modified from the techniques reported by Gessler *et al.*[66] The fluorocarbon used was Genetron, of which 10 cu ml was added to 10 ml. of McIlvane's buffer 1:50, *p*H 7.4, and to this 0.5 to 2 gm of the lymph node to be studied These were mixed in a VirTis No. 23 homogenizer* at 23,000 rpm for 15 minutes, the fluid centrifuged at 1500 rpm for 15 minutes, and the phases separated. The gel phase was removed, 10 ml of buffer was added, and the fluid was again homogenized and centrifuged The aqueous layer of this and the first centrifuging are combined After two further extractions of these combined aqueous layers with 10 cc. of Genetron in the homogenizer the aqueous layer was centrifuged at 40,000 rpm for 6 hours. The resultant pellet was suspended in 0 1 M ammonium acetate All procedures were carried out at 4° C [66]

A total of 8 Genetron extractions have been made using Hodgkin's disease granuloma lymph nodes Four of these revealed round particles of uniform size and shape In three nodes the particles were 25 mμ in diameter and the fourth node 50 to 60 mμ in diameter (FIGURES 9–12) In the normal lymph node tissues, as well as in a lymphosarcoma and some mouse spleens, the [illegible] was 10 mμ in diameter except in one human lymph [illegible] These, however, seemed [illegible] sease material [illegible] shed It should be noted that the particles in three of the Hodgkin's disease materials were of the same order of size as those encountered in the Hodgkin's disease mouse brain material As yet, there is no way of knowing whether these are viruses or some

* Manufactured by the VirTis Company, Inc , Yonkers, N Y



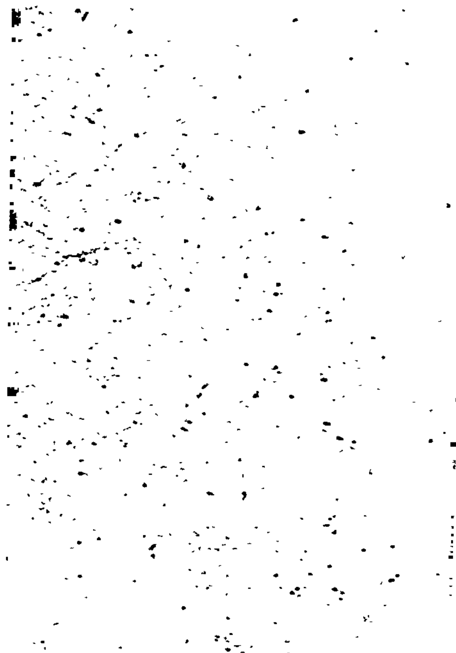
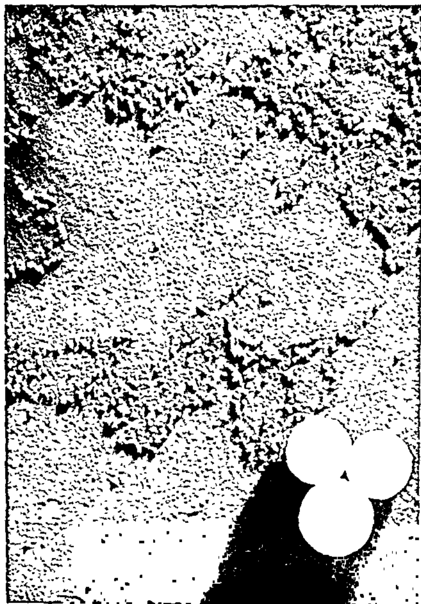


FIGURE 8 Note the absence of the 31 mμ particle seen in FIGURE 7 from this non-Hodgkin's disease control material The small 17 mμ sphere is present in both preparations × 56,000.



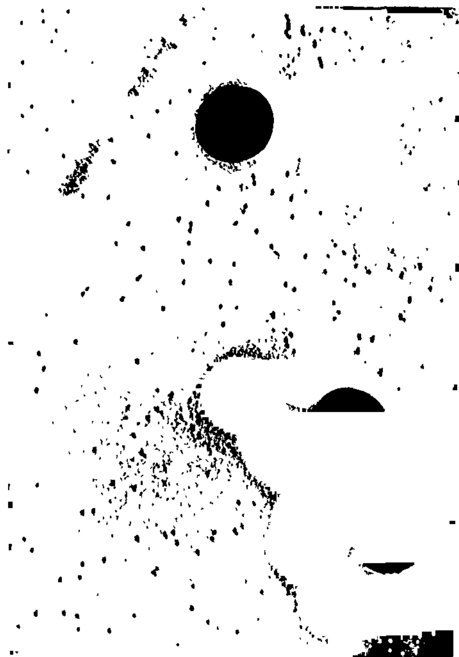
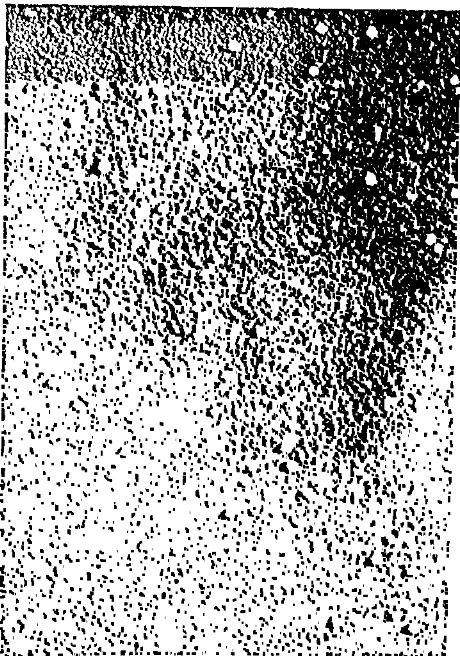


FIGURE 10 Electron micrograph of Hodgkin's disease lymph node material (see caption to FIGURE 9) The larger particles are approximately 25 mμ in diameter × 66,000



FIGURE 11 *Electron micrograph of Hodgkin's disease lymph node material (see caption to* FIGURE 9) The larger particles are approximately 25 mμ in diameter × 66,000



× [illegible]

other intracellular particle that resembles a virus. Some of the Genetron concentrate from the Hodgkin's disease case was inoculated into a culture of HeLa cells with resultant cytopathogenicity apparent in 72 hours. This avenue of exploration and study is being followed. Although the presence of an occasional normal control tissue with roughly similar particles casts doubt on the specificity of the Hodgkin's disease particles, in mouse mammary tumors occasional viruslike particles in supposedly tumor-factor-free animals are found.[20] Such facts as these need further data in order to establish their significance.

Summary

The virus cause of a considerable number of animal malignancies is now well established. The various types of evidence that have been accepted in support of this belief are reviewed in the light of the problems of Hodgkin's disease. It is acknowledged that in Hodgkin's disease some of the desirable criteria will be most difficult to fulfill because of the impossibility of using adequate human studies in a disease of such a fatal nature.

The evaluation of tissue cultures of Hodgkin's disease cells themselves has been most difficult. It is a heterogenous tissue with such great individual variation that objective statements of specific characteristics are elusive. Several workers have reported such things as early liquefaction of substrate and the formation of giant cells and inclusion bodies in such cultures. These changes are not positively very different from those in the controls. The inoculation of Hodgkin's disease extract into heterocellular or organoid tissue cultures has many of the same objections. It is in the use of the pure-line cell culture that the greatest aid can be expected. Various strains of cells are being used. Preliminary experiments indicate that some cytopathogenicity occurs when HeLa cells are inoculated with viral concentrates of Hodgkin's disease tissues.

Animal injections cannot be expected to present much direct evidence. One expects a tumor virus to be very specific in species if evidence derived from the study of known animal virus tumors is valid. In any human tumor virus, unless one by chance finds a natural "carrier" of the virus or demonstrates that a tumor virus, under some circumstances, may not be neoplastic but, instead, inflammatory in type, the use of animals should be disappointing—at least in helping to present direct and convincing evidence of the relationship of the virus under question to tumors of man.

This has been the case in research thus far reported. Serial passages of Hodgkin's disease material in fertile chicken eggs result in an increased mortality of the embryos. When inoculated with Hodgkin's disease material, such eggs possess the ability to interfere with the growth of injected influenza virus. Also, the intracerebral inoculation of newborn mice with Hodgkin's disease material resulted, after several blind passages, in a lethal disease. The disease is clearly caused by a virus agent, many of whose characteristics have been clarified.

As above indicated, such evidence is indirect. Until a virus is related



× 66,000

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As above indicated, such evidence is indirect. Until a virus is related

directly to man and to Hodgkin's disease, a strong reservation must always exist The isolated virus may have come from inoculated eggs, it may be a mouse virus, it may be a human virus unrelated to Hodgkin's disease, or it may be of some other nature. Control experiments have been designed to rule out these possibilities, but they are not absolutely specific Serologic studies, neutralization, complement fixation, and other types of tests can help to relate any virus more directly to the human host. Some such studies have been done; others are planned or in progress

The search for a virus particle per se in Hodgkin's disease material has been extensive. Ever since the papers of Gordon[13] appeared viruslike particles have been seen. However, many of the particles reported by various workers did not resemble each other in size More recently, the size observed has been quite consistent and of the order of 20 to 30 mμ.[44, 55]

These were the particle sizes in the Hoster *et al.*[55] ultracentrifuge studies, of the viruslike particles found in the Hodgkin's disease-injected mouse brain material,[44] and in the purified fluorocarbon extracts that we have made of Hodgkin's disease tissues All of these particles are uniform morphologic spheres Although their characteristics are strongly consistent with those of virus particles, there is no proof that they are actually virus particles, or that they are viable or related to Hodgkin's disease. They represent only a part of the web of circumstantial evidence that can be woven for the virus etiology of Hodgkin's disease.

General research in cancer continually adds evidence in support of the significance of viruses in many kinds of malignancies in animals It cannot be claimed that this has no bearing on human neoplasia In the field of Hodgkin's disease research, the electron microscope and virus purification procedures are adding suggestive evidence of viral origin. These methods, in combination with pure-strain tissue culture techniques and the various aspects of the serologic analysis of antigens and antibodies present, constitute the most promising avenues leading to the ultimate resolution of this deadly disease.

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ETIOLOGICAL CONSIDERATIONS IN HODGKIN'S DISEASE *

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A century and one half of research on Hodgkin's disease has witnessed attempts to implicate each of a variety of bacteria, fungi, and viruses as the etiological agent. The history of this research is a record of hopeful pursuit of false leads ending in frustration, and of complications due to the fact that no laboratory animal has been found to develop the disease either spontaneously or by induction. The voluminous literature has been covered by previous reviewers,[1,2] and those interested in details concerning the disease are referred to the publications listed by them. The present report has to do with those etiological investigations that have at one time and another appeared to be promising and to which the greatest amount of research time has been devoted; namely, those covering the tubercle bacillus, the diphtheroids, and the viruses.

The Tubercle Bacillus

Sternberg[3] was the first to point out a possible relationship between tuberculosis and Hodgkin's disease. The strong points of similarity in both the clinical and pathological pictures and the frequent association of the two diseases in the same patient seemed quite convincing to a number of investigators.

Fraenkel and Much[4] reported Gram-positive granular rods in Hodgkin's disease lymph nodes and believed them to be a form of tubercle bacillus. The failure of many investigators to confirm their results and interpretations did not diminish the flow of reports, both pro and con, on this relationship. The attempt has been made to implicate, as the etiological agent, forms of tubercle bacilli both demonstrable and hypothetical. Inoculation with filtrates from fetal liver and spleen obtained from tuberculous mothers having caused tuberculosis in guinea pigs and "typical Hodgkin's disease" in one animal, Martinolli[5] suggested a filtrable stage of the tubercle bacillus. The granules of Much have been variously interpreted as defatted tubercle bacilli, atypical forms, and as stages in a complicated life cycle that involved a filtrable phase.

L'Esperance[6, 7] reported the isolation of avian tubercle bacilli from animals injected with Hodgkin's disease material and suggested that failure to demonstrate the organism was due to the use of resistant animals. Sensitization of guinea pigs with human tubercle bacilli was reported to render the animals less resistant to the avian organism. The lesions in the lymph nodes were reported to contain cells resembling those described by Sternberg and Reed, and L'Esperance concluded that the accumulated evidence provided proof of the etiological significance of the avian tubercle bacillus in Hodgkin's disease. The report by Stewart and Doan[8] of a high titer in the blood for avian tubercle-phosphatid in twenty-six of thirty-two cases of Hodgkin's disease appeared to support this conclusion.

* The work reported in this paper was supported in part by the Dorothy H. & Lewis Rosenstiel Foundation and The Damon Runyon Memorial Fund for Cancer Research, Inc., New York, N. Y.

Reports contradictory to the tuberculosis theory came from many areas One of the most exhaustive was the investigation by the Rose group[9] Fresh node suspensions from forty Hodgkin's patients and control suspensions from thirty-five patients with a variety of other lymphoid diseases were injected into guinea pigs Material from two Hodgkin's disease patients and two control patients produced tuberculosis in the injected animals The group concluded that "the incidence of tubercle lymphadenoma glands is much the same for controls showing other definite histologic evidence of tubercle "

Equally extensive investigations were conducted by Hoster *et al*[10] Thirty lymph nodes and 3 spleens were obtained from patients with histological evidence of Hodgkin's disease. Smears were made from the fresh tissue, and 6 acid-fast culture media were inoculated with each tissue In addition, 68 guinea pigs, 30 leghorn chickens, and 4 rabbits were given subcutaneous and intravenous inoculations of emulsions of Hodgkin's disease tissue. Controls were inoculated with tissue obtained from patients with other diseases Twenty additional guinea pigs were sensitized with killed human tubercle bacilli 8 to 10 days before inoculation with the tissue emulsion All animals were autopsied 8 months following inoculation No acid-fast organisms were found, and only those animals injected with material from 4 tuberculous control specimens showed tuberculosis The authors concluded that "it is not possible to say that the avian, bovine, or human tubercle bacillus sought under the conditions specified has an etiologic role in Hodgkin's disease "

Thus in two separate extensive and well-controlled experiments the results indicate no relation between the tubercle bacillus and Hodgkin's disease

The Role of Diphtheroids

Extensive investigation and numerous reports on the etiological role of diphtheroids in Hodgkin's disease dominated research during the period from 1911 to 1917. Initially, Bunting and Yates[11-15] reported the isolation of pure cultures of a pleomorphic diphtheroid organism from the nodes of their patients with Hodgkin's disease Confirmatory results were reported by Litterer,[16] by Mellon,[17] by Billings and Rosenow,[18] and by Fox[19]

The organism was described by Bunting and Yates as strikingly pleomorphic, cultures contained long banded and granular rods, fusiform rods, club-shaped and large involutional forms, short, plump bacilli with polar staining, and coccoid forms. The authors later proposed that the term *Corynebacterium hodgkini* be used for this organism They injected monkeys with living organisms and produced "progressive enlargement of a single group of lymph nodes which show histological changes identical with those seen in humans when the disease is of the same duration, namely, chronic lymphadenitis with atypical proliferation of the endothelial cells, beginning proliferation of the stroma, well-marked eosinophilic infiltration and periglandular fibrosis " Continued animal experimentation led the authors to conclude "While the

same time to avoid so great virulence as to produce extensive necrosis, soften-

ing, and even suppuration. The working space between these two limits seems very narrow."

Cunningham[20, 21] criticized the techniques used in the studies implicating the diphtheroids as etiological agents, suggesting that these investigators were "dealing with organisms whose natural habitat is the laboratory." When he used "routine" techniques the organism was isolated from both Hodgkin's and control nodes whereas, with more stringent sterile technique, the organisms could not be isolated from either group of nodes

In summarizing a review of diphtheroids in 1945, Hoster and Dratman[2] concluded that "present day references to the diphtheroids as etiologic agents are made only in historical review; it is generally agreed that there is no evidence for the etiologic relationship between the diphtheroid organism and Hodgkin's disease."

Despite the report of these investigators, the isolation of diphtheroids from the blood of lymphomatous patients was again reported by Fleisher in 1952.[22] Using a culture technique involving several blind passages in tryptose phosphate broth, Fleisher reported one or more positive cultures in 27 of 119 patients Of these 27 patients, those listed as having Hodgkin's disease or lymphomatous tumors had more than one positive culture each; constantly negative cultures were found in 6 cases of various types of leukemia, and cultures from an additional 72 patients with nonlymphomatous disease were all negative After extensive analysis and discussion Fleisher concluded that "when diphtheroid organisms are found on several occasions in the blood cultures of a patient, one should consider the possibility of either Hodgkin's disease or malignant lymphomatous involvement "

Kassel and Rottino[23] became interested in a similar diphtheroid with acid-fast properties that had been found in blood cultures not only of patients with Hodgkin's disease, but of patients with leukemia and lymphosarcoma and of both humans and mice with cancer The organisms appeared as coccoid bodies, rods, and club-shaped forms containing granular Gram-positive inclusions In the rod form the inclusions were bipolar The single consistent feature of the organism was the acid-fast staining property of its inclusions
[illegible] n three cultures supplied
[illegible] and staining character-

The result of several series of cultures fell into no pattern Sporadic positive cultures grew from the blood of patients with Hodgkin's disease, lymphosarcoma, or leukemia, negative results were consistently obtained from one patient and consistently positive results from a few patients whose blood was cultured only a few times, but the great majority alternated between positive and negative

Although these results appeared to be compatible with those of Fleisher, the probability remained that all sources of contamination had not been ruled out. With the help of James Reyniers, of Notre Dame University, Notre Dame, Ind , a small, germfree hood was devised Briefly, the hood was an airtight box, with attached gloves, glass-wool filters to permit the exchange of sterile air, and a chemical trap in which material could be both sterilized

and introduced into the hood. Sterilization techniques were those previously tested and proved by Reyniers' group at the Lobund Institute of the University Blood samples used for cultures were obtained in the operating room with surgical scrub technique and siliconized syringes, and extreme caution was exercised to maintain sterility. Coded samples obtained in quadruplicate were cultured in pairs in the "sterile" room and in the germfree hood as described in a previous report.

The results of this experiment were briefly as follows: of a total of 60 cultures carried in the germfree hood, 3 contaminated cultures were observed during a period in which a break in technique had been discovered, but no cultures were positive for the "diphtheroid" organism previously mentioned. The "contamination" rate in the sterile room was 8+ per cent for the diphtheroid and 8+ per cent for other organisms

Our experience with this organism was that, initially, it was relatively easy to isolate but, as precautions against contamination became more stringent, it appeared in our cultures less and less frequently Finally, when the critical germfree technique was adopted we failed completely to obtain growth, clearly indicating that the "diphtheroid" was a contaminant.

Viral Studies

Discovery by Rous[24] of a virus-induced tumor led to much early speculation as to the viral etiology of neoplasms as a group, and many investigators have suggested that a viral agent might play the etiological role in Hodgkin's disease These opinions, however, were based primarily on clinical observations and on the similarities of some of the pathological manifestations to viral tumors in animals It remained for Gordon and his associates[25-27] to conduct the first systematic investigation of the viral etiology of Hodgkin's disease The results of these investigations require fairly extensive consideration because of the apparent early success achieved by his group

Preliminary inoculation of lymph node emulsions into the rhesus monkey and the mouse gave negative results. Subcutaneous inoculation into the guinea pig resulted in a local nonspecific inflammatory reaction that was noted following a few control injections as well

The rabbit, however, soon became the animal of choice in what is known as the Gordon test The intracerebral inoculation of extracts of Hodgkin's disease nodes into rabbits was followed in 2 to 6 days by a syndrome of muscular rigidity, paralysis, progressive wasting, and death. It was found that 60 to 75 per cent of nodes from Hodgkin's disease patients produced this fatal encephalitis Attempts to isolate bacteria from the brain or blood stream were unsuccessful Control nodes including leukemias, sarcomas, carcinomas, and chronic adenitis gave negative results in all but 2 per cent of cases

Attempts to elucidate the nature of the agent produced the following facts desiccation and resuspension enhanced the potency of the material, the agent resisted freezing and heating to 65° C for 30 minutes, but was inactivated at 100° C for 30 minutes, after centrifugation at 3200 g there was no sedimentation, but the agent was not present following passage through a Chamberland filter, nor could it be passed from an affected to a normal rabbit. Staining

techniques were said to demonstrate elementary bodies of the same order as those of vaccinia and psittacosis It was later found that the syndrome could also be produced in guinea pigs by intracerebral inoculation.

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leukocytes, and pus Turner *et al* [31] and Edwards[32] reported that they could correlate encephalitogenic activity with the presence of eosinophils in the human lesions and bone marrow. Tests with pure leukocytes indicated that

his own cases and those in the literature, McNaught[33] concluded that "the Gordon test is of no more practical value in the diagnosis of Hodgkin's disease than is the finding of eosinophils in the lymph nodes"

Not deterred by these negative results, other investigators have continued the search for evidence of viral reactions Karnofsky *et al* [34] and Bostick[35] observed that extracts of nodes from Hodgkin's disease produced edema in chick embryos, and Hoster and Bechtel[36] and Bostick[37] found that they increased the mortality in embryonated eggs, but not in further serial egg passages. Bostick[35] inoculated similar filtrates into pregnant guinea pigs and guinea pig embryos and found that the fetuses continued to grow, were born, and at autopsy showed no abnormalities Serial intracerebral passages of amniotic fluid extracts into newborn mice were done by Bostick and Hanna,[38] but no clinical or microscopic evidence of disease was observed Kassel and Rottino (unpublished experiments) inoculated cell suspensions and extracts from Hodgkin's disease nodes into more than 500 12- to 16-day-old mouse embryos *in utero*, using both subcutaneous and intracerebral routes Animals brought to weaning were subsequently challenged with the same material, which had been maintained by freezing in dry ice No gross or microscopic evidence of disease was observed.

Following studies of viral interference phenomena Bostick and Hanna[38] continued the intracerebral inoculation of Hodgkin's material and serial blind passage in newborn mice These investigators have recently reported three instances of virus isolation after 5 to 7 blind passages of Hodgkin's disease nodes in suckling mice[39] This virus produced a fatal encephalitis that developed 7 to 10 days after inoculation Initially the virus could be carried only by intracerebral injection, however, after 17 serial intracerebral passages it became adapted to intraperitoneal transfer A great effort has been made to rule out contaminating viruses from the mouse or egg, and these investigators feel that this is a new type of virus that has truly been isolated from human Hodgkin's disease lymph nodes Bostick reports his latest results elsewhere in these pages

Discussion and Summary

The large number of clinical and research reports dealing with Hodgkin's disease attests its highly controversial and speculative nature In the history

of this research there are repeated cycles of association and attempts to implicate one or another etiological agent. Untold energy has been directed toward finding a bacterial causative agent; forty years were required definitely to eliminate the possibility of a relationship between Hodgkin's disease and tuberculosis. The diphtheroid hypothesis, laid to rest by Cunningham in 1917, was revived 35 years later, and an additional four years of effort were required again to prove the diphtheroid to be merely a fastidious contaminant. The false alarms of the "bacteriological era" of Hodgkin's disease research serve to emphasize the extreme refinement of technique required to rule out unusual contaminants The demands are particularly great when a disease of unknown etiology and questionable classification such as Hodgkin's is being considered.

The larger living forms having been laboriously eliminated as etiological agents, it followed quite naturally that submicroscopic particles, the viruses, should next be subjected to study. That the previous difficulties have continued to beset workers in virology is highlighted by the results of Gordon

of the eosinophil and, probably more specifically, to one of its enzymatic components. It is to be expected that, as viral investigation continues, the role of cellular components and contaminating viruses will present even greater problems The contaminants so difficult to eliminate from the bacterial population must have much more numerous counterparts in the viral world

In the face of such difficulties it is not surprising that progress has been slow However, there is no question that the volume of data suggestive of a virus as the etiological agent of Hodgkin's disease has been mounting. The reports of Bostick on the isolation of a virus from patients reinforces this postulate appreciably

A most difficult burden of proof rests upon any investigator who believes that he has isolated a virus from a Hodgkin's disease source for, after having eliminated the possibility of a contaminating virus from the biological system used for the original isolation, he must of course demonstrate that the suspected agent will produce the disease In view of the lack of any susceptible laboratory animal, it is at this time impossible to meet this requirement Hence, it is necessary to find some other response in the laboratory animal that will signal the presence of Hodgkin's-specific material It may be that the mouse paralytic syndrome observed by Bostick will prove to be such an indicator system

In our laboratory, what we have thought to be indicator systems have proved to be inconsistent, nonspecific, and often not reproducible Weight loss in both mice and guinea pigs following inoculation of node suspensions or filtrates was not consistent from one sample to another or in different animals subjected to the same material Sporadic cases of paralysis were observed in guinea pigs inoculated with blood from persons with Hodgkin's disease but, reminiscent of the Gordon phenomenon, this condition was not transmissible from a paralyzed to a normal animal The inoculation of embryo-

nated eggs with blood caused an inconsistent increase in mortality, and deformities and wasting in the animals that survived

Assuming that a virus is the etiological agent in Hodgkin's disease, the manifestation of this virus in a laboratory animal might be prevented by any number of factors such as the presence of inhibitors or modifiers in the virus preparation, virus lability, age-dose relationship, host susceptibility, and route of administration These are but a few of the factors known to play a role in the expression of those tumor viruses most extensively investigated in the past. Any such factors, or any combination of them, could mask the presence of an agent in crude extracts of diseased tissues Bryan and Moloney,[40] discussing these factors in relation to Rous sarcoma, said "the major problem .. appears to be that of separating a relatively small amount of labile virus from overwhelming quantities of nonviral cellular constituents or 'impurities' The ratio of virus to impurities in the best 'partially purified' preparation is estimated to be about 1:100" Methods of preparation alone have been shown to play a critical role in the demonstrable activity, stability, and expression of viruses, and the reports of Burton *et al*[41] indicate that the activ[illegible] increased twelve thou[illegible] that, while the crud[illegible] by the purified preparation are invasive and highly malignant Timing of the inoculation also proved to be of extreme importance, and larvae showed different rates of tumor formation at different ages.

The number of possible combinations of these variables in virus isolation and expression is so overwhelming as to incline one to the belief that the achievement of the proper combination will be purely fortuitous From the extensive studies of groups working on the Rous sarcoma, chicken lymphomatosis and erythroblastosis, rabbit papillomatosis, and mouse mammary tumors and leukemia it is obvious that only fundamental studies will make it possible to sift out the knowledge requisite to the control of these variables When this has been done, a critical analysis of the role of the virus in tumor development may be possible Faced with this problem of searching for an unknown virus that may or may not signal its presence in an experimental animal, our laboratory has chosen an indirect approach. Believing that a more fundamental understanding of mammalian oncogenic viruses may provide the rationale and techniques for attacking human tumors and related syndromes such as Hodgkin's disease, our laboratory has selected mouse leukemia for basic study The work is in its infancy, but it has been possible to reproduce the results previously reported by Gross[42] Even in a comparatively well-known system such as this the initial studies have not been altogether uncomplicated Nevertheless, we are now prepared to test methods of extraction and purification, age-dose relationships in various hosts, and the development of more rapid bioassay techniques It is not anticipated that the carry-over of information and techniques from the model system will be direct, however, it would appear reasonable to expect that techniques tried and tested on such a mammalian system might be applied to human material with success

The task of isolating a pure virus and identifying it beyond question as

the causative agent of Hodgkin's disease is almost overwhelming in its complexity. Every avenue of approach must be tested, and if model mammalian systems do not provide leads it may be necessary to turn to techniques such as tissue culture, or to use even further removed biological systems such as that of *Drosophila*. The search must go on, and even the "negative" results are affirmative in that they warn other investigators away from those approaches that have been shown to lead nowhere.

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THE USE OF GERMFREE ANIMALS AND TECHNIQUES IN THE SEARCH FOR UNKNOWN ETIOLOGICAL AGENTS*

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The subject assigned to us for discussion must be treated, for the most part, theoretically. While the use of germfree techniques is rapidly expanding, the field is relatively new, and very little has been done with either the germfree animal or techniques in the search for unknown etiological agents However, this is a matter of time, so that it is opportune now to discuss the problem.

The thesis upon which germfree life studies rest is that of extending the pure-culture concept to the animal by the use of methods that eliminate contamination (Reyniers, 1953, Reyniers and Trexler, 1953). The value of this concept to the development of bacteriology and infectious diseases is without serious question It would seem reasonable, therefore, that this concept should be extended to situations where an infectious agent is suspected, but unknown, by eliminating contaminants from the animal and the environment in which it lives If these conditions can be achieved, then the animal is locked, in effect, into an isolated environment Under these circumstances attention may be centered on the animal and not diverted by suspicion of contamination from the external environment or from microbes ordinarily contaminating a conventional animal

Theoretically, a germfree animal is one free from all living contaminants Practically, the examination involved requires a function of available techniques for detecting contamination Such techniques will encompass those of bacteriology, virology, parasitology, immunology, and pathology. If the levels of inquiry cannot be satisfied by such methods, then new methods must be devised For example, if a disease occurs in a germfree animal, it may be reasonable to suspect a living contaminant even though conventional techniques do not reveal it Further, if no disease is observed in the germfree animal during its life or in subsequent generations it may be necessary to cause a disease or, better, to bring stress to bear on the animal to bring out a contaminant This would be the case where no spontaneous tumors occur but can be brought about experimentally through the use of a carcinogen.

In the present state of knowledge, it is reasonable to assume, on a basis of experience, that microscopically visible microorganisms (for example, bacteria, yeasts, fungi, protozoa, endoparasites, and ectoparasites) can be eliminated from a mammal by cesarotomy or from oviparous animals by sterilizing the outer shell of the egg and hatching into a sterile environment. The techniques presently available are adequate for the purpose Moreover, it should be remarked that the germfree animal is itself an excellent detection device in which contaminants can readily grow, probably because the animal is without previous microbial experience, and there is no antagonistic effect of one kind

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of microorganism on another. The same may be said for many of the more familiar viruses, especially those that cause a recognizable disease The problem is more complicated with nonsymptomatic viruses Here even the techniques of tissue culture must be refined so that products and tissues from germfree animals may be maintained in a germfree unit This level of investigation has not yet been fully explored. Indeed, it constitutes, in part, the subject of this paper.

At this point it should be remarked that the definition of a germfree animal as one free from all demonstrable living contaminants is correct If, in the search for contaminants below the level of ordinary inquiry, an agent should be revealed, the animal is, of course, not germfree, and further work is necessary to find animals in which it does not exist However, it is at this point that the animal may be of prime interest, since it is isolated with the contaminant, which may be studied without interference from other microorganisms

Germfree Apparatus

Since no degree of asepsis will satisfy the absolute demand of eliminating contamination from the experiment, it is not possible to rear or study germfree [illegible] such apparatus is an essential

[illegible] ather than the term "isolation [illegible] primarily for working with animals isolated from microbes However, any apparatus that will satisfy the rigid standards necessary for rearing germfree animals can also be used in the physical and chemical sciences for controlling the environment at a preselected temperature, humidity, pressure, or gas composition, with slight changes in the accessories

Apparatus designed for rearing germfree animals must be completely sterilized at the start of an experiment and kept sterile during its use It must also permit contaminated materials to be sterilized into it and the individual units to be connected to each other into multiple systems without contaminating the contents Anything less certain than these requirements of absolute sterility will be unsatisfactory for serious work requiring continuous observation over a long period of time

From years of experience with sterilization in microbiology, we know that the most effective way of killing all forms of life is heat, particularly as delivered through steam under pressure (autoclaving) Once used, heat has the added advantage of not interfering with tests for sterility, as might be the case with chemicals

Laboratory tests of the apparatus for sterility that do not involve the animal are of limited value The final test is that of observing the germfree animal for contamination during operation of the equipment In this the germfree animal is the best test medium for the purpose It is the real purpose and the focal point for using the apparatus Laboratory tests are limited, at best, by sampling errors, since only part of the materials (food, water, bedding, and apparatus) can be sampled with specific media Generally speaking, it is best to depend on the method used to effect sterilization rather than a means for

detecting contamination in the case of failure Under such circumstances autoclaving has proved to be the most effective method In special instances where filtration, radiation, or chemical sterilization is necessary, the risk of contamination must be accepted, and the burden of proof lies in detecting it, but wherever possible it would be advisable to use autoclaving In passing, it might be remarked that apparatus designed for steam sterilization also can be used with chemical disinfectant On the other hand, apparatus designed primarily for chemical sterilization cannot be used so effectively with heat.

The basic design requirements for an effective germfree system operated with steam under pressure are as follows:

(1) The apparatus should be constructed of stainless steel and made sufficiently sturdy to withstand continuous use, as well as the thermal stresses and pressures involved in its operation It must be simple enough in construction to permit day-by-day operation in a routine manner without endangering sterility Like an autoclave, it should last for years of constant use and be sufficiently uncomplicated that it can be operated by technicians Thus, the initial costs are not a factor when measured against the demands for a secure operation.

(2) The apparatus should consist of basic units that can be used singly or assembled into systems specialized to certain specific tasks (FIGURE 1).

(3) Each unit must have such flexible members as rubber gloves attached to it so that the animals can be manipulated

(4) Each unit must have a means to permit *in situ* sterilization, so that food and water may be passed into the unit, debris removed from it, or two or more units connected for transfer of animals without breaking sterility.

(5) Each unit must be supplied with a ventilating system capable of delivering sterile air to it

(6) Each unit must be equipped with viewing windows and lights

One such system that meets the above requirements is the Reyniers Germfree System * The basic unit is a metal cylinder, preferably made of stainless steel (FIGURE 2) Each unit is mounted on wheels so that it can be moved freely. A complete germfree system in which germfree animals can be reared, experimented upon, and cesarotomized would consist of the following specialized units (1) a standard rearing unit, for example, RSU-500, (2) a surgical unit, ROPU-200 in which cesarotomy can be performed on conventional mammals, (3) a unit, REXU-200, in which animals can be experimented upon and that also serves as a connecting unit for end-to-end systems, and (4) transport units, RTRU-100, in which animals may be shipped from place to place via public transportation

The numbers of each type of unit necessary to an operation will depend on the needs of the experiment and the numbers of animals necessary If ani-

* Reyniers & Son, 3806 North Ashland Avenue, Chicago, Ill The code numbers are those of this firm

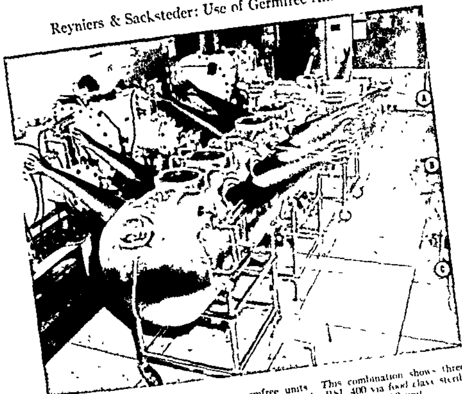


FIGURE 1 Combination of Reyniers germfree units This combination shows three units (A) RSU-400 unit, (B) REXU-200s connected to the RSU 400 via food clave sterile lock and to REXU 200 unit by end to-end connection, and (C) REXU 200 unit

freeze units, homogenizers, storage units, auxiliary food claves, and other such items, are also available so that the germfree system can be adapted to many experimental needs

A germfree unit is operated by first making certain that it does not leak at gasket surfaces, then covering it with an insulating blanket and sterilizing it at 254° F. for 30 minutes, after which it may be dried at a negative pressure of 20" Hg Animals may be introduced into the sterilized unit either by attaching it to a unit already containing germfree animals or to a surgical unit if the animals are to be obtained by cesarotomy The linkage between the two units is sterilized by autoclaving, after which a transfer can be made Food and other materials may be passed into the germfree unit by placing them into the sterile lock or food autoclave and autoclaving *in situ* Following this, the inner door of the lock may be opened and materials taken into the unit Heat is kept from the animals during the sterilization cycle of a food clave by covering the inner door with an insulating cap and running a stream of water around the outside of the shell of the unit where it joins the food clave

Equipment such as has been described may be used with certainty For example, a single unit has been in full operation with germfree mice for more

detecting contamination in the case of failure. Under such circumstances autoclaving has proved to be the most effective method. In special instances where filtration, radiation, or chemical sterilization is necessary, the risk of contamination must be accepted, and the burden of proof lies in detecting it, but wherever possible it would be advisable to use autoclaving. In passing, it might be remarked that apparatus designed for steam sterilization also can be used with chemical disinfectant. On the other hand, apparatus designed primarily for chemical sterilization cannot be used so effectively with heat.

The basic design requirements for an effective germfree system operated with steam under pressure are as follows:

(1) The apparatus should be constructed of stainless steel and made sufficiently sturdy to withstand continuous use, as well as the thermal stresses and pressures involved in its operation. It must be simple enough in construction to permit day-by-day operation in a routine manner without endangering sterility. Like an autoclave, it should last for years of constant use and be sufficiently uncomplicated that it can be operated by technicians. Thus, the initial costs are not a factor when measured against the demands for a secure operation.

(2) The apparatus should consist of basic units that can be used singly or assembled into systems specialized to certain specific tasks (FIGURE 1).

(3) Each unit must have such flexible members as rubber gloves attached to it so that the animals can be manipulated.

(4) Each unit must have a means to permit *in situ* sterilization, so that food and water may be passed into the unit, debris removed from it, or two or more units connected for transfer of animals without breaking sterility.

(5) Each unit must be supplied with a ventilating system capable of delivering sterile air to it.

(6) Each unit must be equipped with viewing windows and lights.

One such system that meets the above requirements is the Reyniers Germfree System.* The basic unit is a metal cylinder, preferably made of stainless steel (FIGURE 2). Each unit is mounted on wheels so that it can be moved freely. A complete germfree system in which germfree animals can be reared, experimented upon, and cesarotomized would consist of the following specialized units: (1) a standard rearing unit, for example, RSU-500, (2) a surgical unit, ROPU-200 in which cesarotomy can be performed on conventional mammals, (3) a unit, REXU-200, in which animals can be experimented upon and that also serves as a connecting unit for end-to-end systems, and (4) transport units, RTRU-100, in which animals may be shipped from place to place via public transportation.

The numbers of each type of unit necessary to an operation will depend on the needs of the experiment and the numbers of animals necessary. If animals can be obtained from a central source of supply, a single unit may suffice.

There are many accessory attachments such as sterile traps for passing heat-labile materials or embryonated eggs into a germfree unit. Incubators, deep-

* Reyniers & Son, 3806 North Ashland Avenue, Chicago, Ill. The code numbers are those of this firm.

than 15 months without transferring the animals and, during this period, more than 135 sterile lock passages have been made without contamination. A colony of mice has been reared for more than 8 generations, each up to 900 days of age.

It is possible to devise many types of systems for sterilizing air, but long experience has demonstrated that, by the use of fresh, prefiltered outside air passed through glass fiber filters attached to the germfree unit, no difficulty due to contamination has been encountered. The air filters attached to the germfree unit are made to be sterilized *in situ* by autoclaving and dried in heat under vacuum. If, however, for theoretical reasons additional precautions seem necessary, the air may be presterilized with heat and cooled before passing it through the unit filters. This is merely a matter of refinement and does not affect the basic unit or its design.

The point is that, with a basic metal unit that can be initially sterilized with steam under pressure and so maintained in operation, it is entirely possible to work with certainty and to do this with technicians, so that the cost of operation is reasonable. Having such apparatus, it is possible then to concentrate on the animal in the search for unknown etiological agents.

Germfree Animals

Thus far there has been no known certain means of obtaining germfree animals except to remove them before birth to a sterile environment. At this time, and on a basis of many years' experience, it is entirely reasonable to state that chick[illegible] monkeys, goats, dogs

tainty with respect to nonsymptomatic viruses, and this state will always exist because, as such viruses are demonstrated, they are eliminated from the category of the unknown. When this happens, still others may exist for which tests are not available. Finally, there very well may be a level at which the virus may be so closely related to the economy of the cell that it may be necessary to its function. At the moment this is a theoretical question that may

Figure 2. [illegible]

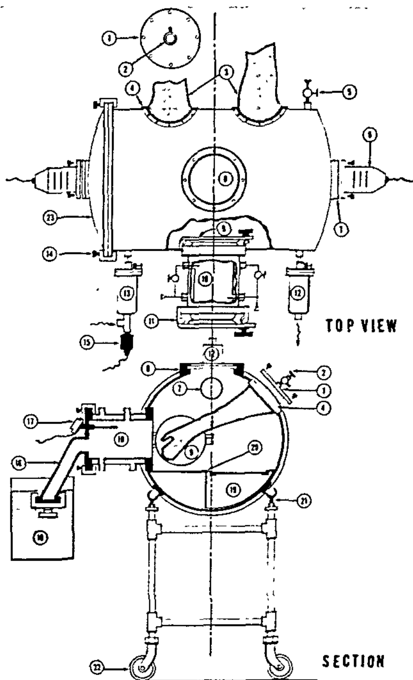

TOP VIEW
SECTION

TABLE 1

EFFECT OF UF* FILTRATES OF HUMAN SERA FROM PATIENTS WITH INFECTIOUS HEPATITIS WHEN INJECTED INTO GERMFREE CHICKENS, LOBUND [illegible]

No. birds	[illegible]	[illegible]	[illegible]	Variety of chicken	Remarks
[illegible]	[illegible]	[illegible]	[illegible]	Leghorn	One bird questionable
[illegible]	[illegible]	—	[illegible]	Leghorn	
[illegible]	[illegible]	—	[illegible]	N. H. Red	
[illegible]	[illegible]	—	[illegible]	Leghorn	Conventional birds
[illegible]	[illegible]	—	[illegible]	Leghorn	Heat inactivated sera
[illegible]	[illegible]	[illegible]	—	Leghorn	

* UF is an ultrafine Pyrex fritted glass filter with a maximum pore size of 0.9 to 1.4 μ.

meticulous attention to technique, short of the use of the germfree bowl, served only to eliminate the commoner contaminants leaving behind slower-growing, more fastidious contaminating organisms of unknown origin and significance. The question of the origin of such an organism is always a critical one, particularly when one is working with material from a disease of unknown etiology and questionable classification such as Hodgkin's."

Again, as the authors point out, this does not settle the question of virus etiology. Here the problem enters another phase, for the germfree technique is only as exact as the purity of the material introduced into the system. Therefore, even when extraneous contaminants are eliminated, the blood taken from conventional animals or patients would be locked into the system with whatever viruses or other contaminants it might contain. The use, therefore, of blood or tissues from conventional animals will always constitute a problem when they are introduced into a germfree system.

An example of this difficulty occurred in some of our work with infectious hepatitis. This is mentioned here only because it illustrates the general problem. In this work we attempted to examine hepatitis sera from the Akiba (Pa.) and New Lisbon (N. J.) pools.* The purpose was to determine whether a laboratory animal could be found that would be useful in experimental studies.

Germfree chickens (White Leghorn and New Hampshire Red varieties) were used in the preliminary experiments because these animals were available at the time. The serum was passed through UF fritted glass filters (0.9 to 1.4 μ) and tested free from bacteria. This was further borne out by the absence of bacterial contamination in the germfree animals. The route of infection was intravenous, intramuscular, or oral. The results of these preliminary experiments are shown in TABLE 1.

Illness developed in the germfree chickens between 9 and 21 days postinoculation. The principal symptoms were gastrointestinal, with marked distention in the crop, which was filled with a foamy liquid. External evidence of this was seen in the discharges from the mouth and nasal passages. At this point in the syndrome, extreme weakness, followed by paralysis and death, was

* The sera were supplied by Warren Henle and Joseph Stokes, Children's Hospital, University of Pennsylvania, Philadelphia, Pa.

eventually be of considerable importance, but only time and a continual re-evaluation of the question can solve it.

The important thing to consider is that an animal is locked into a sterile environment and contains only that which it brings into it Nothing is added from the outside environment, nor is there the uncertainty that would exist, for example, if attempts had been made to eliminate contamination from a conventional animal. Under such circumstances it is possible to search for suspected but unknown living agents as the animal passes through its life into successive generations. In the event that a balanced situation exists between viruslike agents and the animal, it may be possible to upset this balance by placing stress on the animal, with the result that the contaminant emerges to

within the confines

rom a conventional animal, there is little certainty that it is free from contaminants By the same token, in testing conventional animal sera for contaminants in a germfree animal, there is always the possibility of passing unwanted viruses even though the serum is filtered. The use of antibiotics does not satisfy the rigid requirements set up for germfree operations, and this is also true for germicides because of a possible bacteriostasis. On the other hand, the certainty is increased if the tissues and serum are taken from germfree animals and all culturing takes place inside a germfree system

These things are difficult to explain to those not experienced with germfree techniques and concepts They can be explained better with a few examples taken from our own experience and the experience of others working in this field

Use of Germfree Techniques and Animals in the Search for Unknown Etiological Agents

No matter how carefully an investigator works with aseptic techniques the question of contamination is always present. The early period of bacteriology, when the question of life cycles of bacteria was an important problem, is replete with examples of findings upon which the doubt of contamination could be cast, despite very careful work

Use of germfree techniques to eliminate contamination A good example of the need for germfree techniques in cancer research is found in the work of Kassel and Rottino (1955) dealing with the significance of diphtheroids in malignant disease These investigators report the isolation of acid-fast and diptheroid microorganisms from the blood of patients with Hodgkin's disease, leukemia, and lymphosarcoma, as *well* as from mice with cancer *With* conventional aseptic procedures, 20 per cent of the blood cultured from Hodgkin's disease patients on tryptose broth showed diphtheroid contamination and, with PPLO-media, 92 per cent of the samples showed contamination When the work was carried on in a "sterile" room, 44 per cent contamination occurred with patients and 30 per cent with C3H mice. Use of a simple germfree apparatus reduced the contamination to 0 per cent in all instances Thus, the diphtheroids are clearly contaminants The authors say, "In our work

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Age (days)	Incidence of syndrome	Incubation period (days)	Period of observation	Variety chickens	Remarks
30–60	5/6	9–20	60	Leghorn	One bird questionable
8–10	0/10	—	60	Leghorn	
30	0/5	—	30	N H Reds	
30	0/12	—	60	Leghorn	Conventional birds
30	0/12	—	60	Leghorn	Heat inactivated sera
60	8/8	12–19	—	Leghorn	

* UF is an ultrafine Pyrex fritted glass filter with a maximum pore size of 0 9 to 1 4 μ

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TABLE 2

PASSAGE IN GERMFREE CHICKENS VIA SERUM FROM THOSE INOCULATED WITH UF FILTRATE OF HUMAN INFECTIOUS HEPATITIS SERUM (AKIBA POOL)

Age (days)	Passage number	Incidence of disease	Incubation period (days)	Period of observation	Variety chickens
76	I	3/3	12-20	30	Leghorn
88	II	3/3	10-12	30	Leghorn
58	III	3/4	2-8	60	Leghorn
60	IV	0/3	—	60	N H Red

the usual picture. On gross post-mortem examination, no liver damage was detected. The incidence of this disease was erratic, that is to say, when the disease occurred, all chickens in the group were affected, but in other situations none of the birds so inoculated showed symptoms. The best results were obtained with older chickens (30 to 60 days), while the results were uniformly negative in young chickens (8 to 10 days). The Leghorn seems susceptible to the disease, while the New Hampshire Red chicken was uniformly negative. The disease can be transmitted (TABLE 2) through at least three passages in Leghorn chickens In the experiment mentioned the fourth passage was attempted in New Hampshire Red chickens from the Leghorns, and the results were negative We could not reproduce this disease in conventional White Leghorn chickens at any age Heat-inactivated sera were negative Attempts at serologic identification were unreliable

Based on epidemiological and human volunteer studies, it is generally accepted that infectious hepatitis has a virus etiology, and that attempts to find a suitable laboratory animal have been unsatisfactory. Therefore, at this point in our investigations, short of human volunteer studies an impasse was reached. It is obvious that in this work, starting with human sera from active hepatitis patients, other viruses might also be present Since, in the disease as manifested in the germfree chicken, no hepatic damage was observed and paralysis was a symptom, further testing of the serum in human volunteers was not indicated

Here then, within the extremely narrow limits of these preliminary studies, an infective filter-passing agent could be properly suspected, but not identified, and the use of conventional sera failed to meet the level of control necessary Further work on purifying the sera, that is, to eliminate other viruses, is necessary This can be done, of course, by taking advantage of the resistance of different viruses to heat, chemicals, or radiation, or by such means as differential centrifugation

Search for etiological agents in diseases that occur spontaneously in germfree animals, but not in the conventional counterparts The problem here is different from those previously mentioned in that the search is confined entirely to the [illegible] nentally attempted from

[illegible] marked by brain lesions in a series of chickens shortly after hatching. More than one hundred animals were involved This syndrome occurred only in germfree chickens and not

in chickens from the same clutch of eggs hatched into a conventional environment. The symptoms first appeared twenty-four hours after hatching as a fine tremor that progressed over a five-day period to very coarse tremors, lack of coordination, and death. Eventually, all the chickens in the germfree unit where it was detected showed symptoms and, with two exceptions, all chickens allowed to come to termination died. The pathology (studied by H. A. Gordon of our staff) was marked by brain lesions that in one period were characterized by meningeal proliferation in the region of the cerebellum and in another by cerebral lytic areas. In a few instances where the animals were contaminated with bacteria early in the onset of the disease, the lesions were arrested, and the chickens recovered and lived. Attempts to transmit the disease experimentally to conventional chickens were uniformly negative, as were attempts to grow anything from filtrates into the chick embryo. An attempt by M. [illegible]

[illegible] milder and took about seven days to develop. One thing seems certain: the disease occurred only in the germfree animal insofar as our experience is concerned. While there is no direct evidence for an infective agent, the pattern of spread in a group of chickens within the same germfree unit suggests that an unknown etiological agent is involved. Since the disease could not be demonstrated either spontaneously or by inoculation into conventional chickens from the same clutch of eggs, the problem, if it is to be properly explored, lies within the confines of the germfree system.

This strange disease of germfree chickens has not been fully worked out because of the sporadic occurrence of the malady and the difficulty of predicting its appearance, so that disease-free, germfree chickens would be available for transmission studies.

Possible transmission of etiological agents from conventional parents to offspring reared germfree. We are here mostly concerned with suspected but unknown etiological agents such as would be found in egg transfer in avian leukosis, leukemia in AKR mice and, to some degree, in transmission of the Bittner factor in C3H mice. In order to illustrate the problem within the framework of germfree animals and techniques, an example is given from our work with germfree C3H mice.

Because germfree mice are cesarian born and are hand fed to weaning on an autoclaved liquid formula or foster-suckled on germfree mothers that themselves have been hand reared from a cesarotomy, it is reasonable to assume that transmission of the milk factor via mother's milk would be eliminated. Therefore, if spontaneous tumors develop, the possible factor would probably be transmitted to the offspring through the placenta or by way of the germ cells. If such agents are transmitted through the placental barrier, it would be reasonable to expect tumors to develop in the first generation, that is, in hand-fed animals. If tumors are manifested in succeeding generations, then it may b[illegible]
is, genet[illegible]
strain ar[illegible]

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above, and where it is possible that the etiological agent might be dormant. At this point attempts can be made to stimulate tumors by a carcinogen and to search for transmissible subcellular particulates in the tumor. Since this can be accomplished within the confines of a germfree system and in an animal that is usually negative to the spontaneous disease, the search assumes a less complicated situation than with conventional animals.

An example of the use of a diet to bring about a pathology where an unknown etiological agent might be suspected concerns liver necrosis in rats In a series of experiments with germfree rats fed a diet that will cause liver necrosis in conventional animals (Luckey *et al*, 1954), the first results were negative in the germfree animal Another series of germfree rats in which the diet was restricted yielded liver necrosis. Because the use of antibiotics and chemotherapeutic drugs gave protection to conventional rats (Gyorgy, 1954) fed a [illegible]

curred, isolation and passage of a subcellular agent were attempted by passages to other germfree animals. The results have been negative to date in these preliminary experiments, but the experiment illustrates the category of usefulness now under consideration

Summary

The purpose of this discussion has been to introduce the subject of germfree life and techniques in the isolation of unknown etiological agents While the animals and the techniques are available for the purpose, the field is new, and not much has been done in this respect Therefore the discussion has been held largely at a theoretical level.

The basis for such usefulness lies in an extension of the pure-culture concept through the use of germfree animals and techniques Since the germfree animal is free from demonstrable living contaminants and in a sterile environment, the question of contamination is negligible [illegible]

the animal, the experiment has little chance of success

Given the proper apparatus and the germfree animal, it is possible (1) to use the apparatus to eliminate contamination from microbiological culturing techniques, as in the search for blood parasites (Kassel and Rottino, 1955) or to use the animals in an attempt to isolate and grow the virus of infectious hepatitis, (2) to search for etiological agents in diseases that occur spontaneously in germfree animals, (3) to study the transmission of disease-causing agents from conventional animals to germfree offspring, and (4) to search for unknown etiological agents in diseases caused experimentally by stress, chemicals, or diet in germfree animals

From the above an attempt has been made to show the usefulness of the hypothesis to various fields of interest, including cancer research

TABLE 3
GERMFREE C3H MICE
JULY 27, 1955, TO MAY 30, 1957

Generation	Number of animals started	Sex		Age in days to May 30, 1957	No under observation May 30, 1957	
		♂	♀		♂	♀
First	7	1	6	655	1	1
Second	46	28	18	520	6	4
Third	98	51	47	485	35	24
Fourth	81	42	39	390	27	19
Fifth	61	32	29	330	33	26
Sixth	20	9	11	250	8	10
Totals	313	163	150		110	84

from the male Finally, if no tumors develop spontaneously, either in the lifetime of the animal or in successive generations it may be said that such a factor is absent or at least dormant and waiting for some stress not found in the normal life of the animal or some genetic accident to free it These are simple hypotheses, of course, and are given to illustrate the problem under consideration

An experiment set up to test these hypotheses and not yet completed may be offered as an example In a group of C3H mice (Tannenbaum substrain) born by cesarotomy and delivered germfree, the animals were foster-suckled on a second-generation germfree Swiss mother that had delivered naturally on the same day The Swiss strain concerned is conventionally a low tumor-incident strain The C3H animals were weaned and bred brother to sister for seven generations Some animals from each generation were allowed to live out their life span All animals were observed for tumors The results in the time period indicated are shown in TABLE 3

As can be seen from the results, none of the original litter of C3H mice developed tumors, nor were tumors observed in the offspring of the seventh generation

Conventional C3H mice on the same autoclaved diet and reared in the same type of container show 90 per cent mammary carcinoma between the tenth to eleventh month and 98 per cent by the eighteenth month The conventional males develop about 45 to 50 per cent hepatomas by the thirteenth month

Since this is an interim experiment and the group is small, a note of caution should be sounded relative to conclusions It might be expected with a greater number of animals and over a longer period of observation that tumors would develop in the germfree strain, at which point attempts to isolate an agent could be made

Search for suspected etiological agents in diseases caused by stress, chemicals, or diet in germfree animals Another example of the possible usefulness of germfree techniques and animals is that in which an anticipated disease does not occur spontaneously, for example, tumors in germfree C3H mice as mentioned

Part IV. Therapy

RESULTS OF THERAPY IN HODGKIN'S DISEASE*

By Henry D Diamond

The Memorial Center for Cancer and Allied Diseases, Sloan-Kettering Institute for Cancer Research, and Cornell University Medical College, New York, N Y

Since 1950, a detailed study of all patients with Hodgkin's disease observed at The Memorial Center for Cancer and Allied Diseases has been in progress. Lloyd F. Craver and I, in collaboration with the late Sophie Spitz of our Department of Pathology, undertook the task of studying the clinical records, pathology, clinical course, treatment, and prognosis of those patients in the records of this center from 1918 through 1953 In addition, my private practice records and those of Craver were perused and analyzed to augment the hospital records

As a consequence of this plan, and in order to implement the investigation, a master code chart was devised with the aid of our statistical department, and the data were transferred to IBM cards for processing through IBM machines Using such techniques, we were able first to extract and then to analyze considerable masses of data on our patients with Hodgkin's disease

We found almost 2500 records of patients with this disability. About 500 of the cases recorded from 1918 through 1928 were not verified by biopsy or other histopathological studies Of the remaining 2000 cases, all with histopathological confirmatory studies, almost 1000 were either brief consultation cases or, for various reasons, had insufficient data recorded on the charts to meet the rigid criteria formulated for this investigation Although each of these 2000 cases had been diagnosed as Hodgkin's disease on slide study by some member of our Department of Pathology, it was decided that a single member, Sophie Spitz, should review all the pathological material The re-

again reduced the total study group Thus, by December 31, 1953, the closing date of this clinical investigation, we were able to assemble a grand total of 713 records of patients with Hodgkin's disease that met the following rigid criteria of our study (1) completely adequate clinical records, so that all ques-

therapy, or surgery

As other investigators have done,[1-4] we have evolved a clinical classification of Hodgkin's disease that serves a triple function first, it allows us to consider

* The research for this paper was supported by a grant from the Lloyd F Craver Fund of The Memorial Center for Cancer and Allied Diseases, New York, N Y

Acknowledgments

We acknowledge gratefully the assistance of Robert Sieczko, who has operated the germfree equipment used in these studies, and also the assistance of Erwin Zelmer and Bernard Teah

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ermfree research a basic study in the host-
theoretical aspects of the problem Bull

TABLE 1

5- AND 10-YEAR SURVIVAL RATES BY DECADES IN 713 PATIENTS WITH HODGKIN'S DISEASE

Decade	5 Years	10 Years	Mean duration in years from 1st Memorial Center visit
1st (0–9 yrs)	1/11 = 9 0%	0/11 = 0 0%	2 4
2nd (10–19 yrs)	18/81 = 22 2%	8/81 = 9 9%	3 4
3rd (20–29 yrs)	38/188 = 20 5%	14/188 = 7 4%	3 1
4th (30–39 yrs)	37/174 = 21 3%	16/174 = 9 2%	3 3
5th (40–49 yrs)	25/110 = 22 7%	5/110 = 4 5%	2 8
6th (50–59 yrs)	14/82 = 17 1%	5/82 = 6 1%	2 8
7th (60–69 yrs)	3/46 = 6 5%	2/46 = 4 3%	1 8
8th (70–79 yrs)	3/20 = 15 0%	1/20 = 5 0%	2 2
9th (80–89 yrs)	0/1 = 0 0%	0/1 = 0 0%	0 8

tient survived beyond the 5-year period, making for this group a 9 per cent 5-year figure

In the second decade there were 81 patients with a mean duration of 3 4 years, a 5-year survival rate of 22 2 per cent (18 of 81 cases), and a 10-year survival rate of 9 9 per cent (8 of 81 cases) Obviously only some of the patients of the 5-year group survived the period of 10 or more years, but it may be significant that of 18 patients who survived for 5 years, almost half survived for 10 years or more This group includes several who are surviving beyond a 20-year period

There were 188 patients in the third decade with a mean survival of 3 1 years, a 5-year survival rate of 20 5 per cent (38 of 188 cases), and a 10-year survival rate of 7 4 per cent (14 of 188 cases) There are 4 patients in this group surviving beyond the 20-year period

In the fourth decade there were 174 patients whose mean duration was 3 3 years, a 5-year survival rate of 21 3 per cent (37 of 174 cases), and a 10-year survival rate of 9 2 per cent (16 of 174 cases) Three patients in this group are beyond the 20-year period of survival

There were 110 patients in the fifth decade with an average survival of 2 8 years, a 5-year survival rate of 22 7 per cent (25 of 110 cases), and a 10-year survival rate of 4 5 per cent (5 of 110 cases) Thus, 20 per cent of the 5-year survivors lived beyond the 10-year period

In the sixth decade there were 82 patients with a mean survival of 2 8 years, a 5-year survival rate of 17 1 per cent (14 of 82 cases), and a 10-year survival rate of 6 1 per cent (5 of 82 cases) Thirty six per cent of the 5-year survivors lived beyond a 10-year period

There were 46 patients in the seventh decade with an average duration of 1 8 years, a 5-year survival rate of 6 5 per cent (3 of 46 cases), and a 10-year survival rate of 4 3 per cent (2 of 46 cases) Sixty-six per cent of the 5-year survivors lived beyond the 10-year period

In the eighth decade 20 patients had a mean survival of 2 2 years, a 5-year survival rate of 15 per cent (3 of 20 cases), and a 10 year survival rate of 5

treatment either from a theoretically curative standpoint, or from a solely palliative one, second, it places precise emphasis on the choice of one or another treatment agent or agents; and third, it affords us a means (not always altogether satisfactory) of attempting prognosis with some degree of accuracy.

Class 1 The disease is limited clinically to a single locus (unifocal unicentric origin) with no constitutional symptoms or signs, such as fever, night sweats, pruritus, weight loss, anemia, or fatigue

Class 2. The disease is limited regionally (regional anatomic distribution, that is, all apparent disease [illegible]

[illegible]

[illegible] and signs.

This clinical classification[5, 6] concerns itself with the question of whether, when first seen, the patient has localized, regional, or generalized disease. To date, our *impression* is strong that by utilizing the implications of this classification the end results of therapy are enhanced, and the principles of treatment given a discipline Peters,[3, 4] using a like plan, has reported remarkable end results in terms of five- and ten-year survival rates by using X-ray treatment plans, fields, and factors similar to ours

Our 713 patients were treated with therapeutic X rays and with the two polyfunctional alkylating compounds, nitrogen mustard (HN2) and triethylene-melamine (TEM) We have not as yet analyzed our patients either in terms of specific type of Hodgkin's disease (that is, paragranuloma, granuloma, and sarcoma) with respect to preferential outcome, or by a clinical classification breakdown, although figures for doing so will become available to us in the near future; we think these figures will substantiate our present impressions In this report we can discuss only our over-all total experience in 713 cases.

It must be stated at this point that more than 50 per cent of the patients in this group came to us not only in an advanced stage of the disease, but also after having received initial and subsequent treatment in other institutions and by other physicians Regrettably, all too often our services have been sought as a last resort Obviously, this has weighted our case load with an inordinate number of patients who responded unfavorably to treatment and who, therefore, could be expected to have a depleting effect on over-all end results

We analyzed our 713 patients in terms of incidence-frequency distribution according to decades The highest incidence was in young adults, more than one half (362) of the patients were between the ages of 20 and 39 years when first seen and diagnosed at Memorial Center The mean survival time for the entire group of 713 patients was 2 5 years from the time of diagnosis at first visit Our data suggest that the older age group and very old people with Hodgkin's disease had a shorter-than average survival, but then, they were subject to many factors other than Hodgkin's disease that might have shortened their survival

As shown in TABLE 1, for the 11 patients in the first decade of life, the average survival was 2.4 years, with a range of from 10 to 88 months Only one pa-

TABLE 1

5- AND 10-YEAR SURVIVAL RATES BY DECADES IN 713 PATIENTS WITH HODGKIN'S DISEASE

Decade	5 Years	10 Years	Mean duration in years from 1st Memorial Center visit
1st (0–9 yrs)	1/11 = 9.0%	0/11 = 0.0%	2.4
2nd (10–19 yrs)	18/81 = 22.2%	8/81 = 9.9%	3.4
3rd (20–29 yrs)	38/188 = 20.5%	14/188 = 7.4%	3.1
4th (30–39 yrs)	37/174 = 21.3%	16/174 = 9.2%	3.3
5th (40–49 yrs)	25/110 = 22.7%	5/110 = 4.5%	2.8
6th (50–59 yrs)	14/82 = 17.1%	5/82 = 6.1%	2.8
7th (60–69 yrs)	3/46 = 6.5%	2/46 = 4.3%	1.8
8th (70–79 yrs)	3/20 = 15.0%	1/20 = 5.0%	2.2
9th (80–89 yrs)	0/1 = 0.0%	0/1 = 0.0%	0.8

tient survived beyond the 5-year period, making for this group a 9 per cent 5-year figure

In the second decade there were 81 patients with a mean duration of 3.4 years, a 5-year survival rate of 22.2 per cent (18 of 81 cases), and a 10-year survival rate of 9.9 per cent (8 of 81 cases). Obviously only some of the patients of the 5-year group survived the period of 10 or more years, but it may be significant that of 18 patients who survived for 5 years, almost half survived for 10 years or more. This group includes several who are surviving beyond a 20-year period

There were 188 patients in the third decade with a mean survival of 3.1 years, a 5-year survival rate of 20.5 per cent (38 of 188 cases), and a 10-year survival rate of 7.4 per cent (14 of 188 cases). There are 4 patients in this group surviving beyond the 20-year period

In the fourth decade there were 174 patients whose mean duration was 3.3 years, a 5-year survival rate of 21.3 per cent (37 of 174 cases), and a 10-year survival rate of 9.2 per cent (16 of 174 cases) Three patients in this group

survival rate of 4.5 per cent (5 of 110 cases) Thus, 20 per cent of the 5-year survivors lived beyond the 10-year period

In the sixth decade there were 82 patients with a mean survival of 2.8 years, a 5-year survival rate of 17.1 per cent (14 of 82 cases); and a 10-year survival

er cent of the 5-year survivors

with an average duration of 1.8 years, a 5-year survival rate of 6.5 per cent (3 of 46 cases), and a 10-year survival rate of 4.3 per cent (2 of 46 cases) Sixty-six per cent of the 5-year survivors lived beyond the 10-year period.

In the eighth decade 20 patients had a mean survival of 2.2 years, a 5-year survival rate of 15 per cent (3 of 20 cases), and a 10-year survival rate of 5

TABLE 2
METHODS OF THERAPY IN 713 PATIENTS WITH HODGKIN'S DISEASE

X rays HN2/TEM	713 Patients 155/713 Patients
X rays	Spark-gap 200 kv. 250 kv. 1 Mev
HN2/TEM	Multiple courses in many patients

per cent (1 of 20 cases) One of the three 5-year survivors lived beyond the 10-year period

We had only 1 patient in the ninth decade, and this patient survived only 10 months

All 713 patients were treated with X rays. In the period from 1918 to 1928 dosage calculations were based on spark-gap measurements, and for this reason are difficult to measure and assess From 1928 to 1936 treatment was given on high-voltage equipment ordinarily generating 200 kv. From 1936 through 1953 all patients were treated on 250 kv. generators, and some received super-voltage therapy on the 1-Mev machine as well. We have not as yet analyzed whether these different modalities have different effects on survival times (TABLE 2).

Of the group of 713 patients, 155 were given one or more courses of either HN2 or TEM. The effects, if any, of these chemotherapeutic agents on survival time have not yet been analyzed

The over-all 5-year survival rate for the entire group of 713 patients was 19 5 per cent (139 of 713 cases), and 7 2 per cent was the 10-year survival rate (51 of 713 cases), as shown in TABLE 3 Of the 5-year survivors, 36 8 per cent (51 of 139 cases) lived beyond the 10-year period. Moreover, a considerable number of patients are now living and well beyond the 20-year period In 1957 a number of patients were living and well beyond the 25-year period

TABLE 3
OVER ALL 5 AND 10-YEAR SURVIVAL RATES IN 713 CASES OF HODGKIN'S DISEASE

Decade	5 Years	10 Years
1	1	0
2	18	8
3	38	14
4	37	16
5	25	5
6	14	5
7	3	2
8	3	1
9	0	0
	139 = 19 5%	51 = 7 2%

Although the over-all 5-year survival rate seems discouraging, it must be kept in mind. (1) that all patients from those in the most advanced stages to those in the earliest stages of the disease have been included without selection in the study; (2) that our study group is weighted heavily (more than 50 per cent) with patients who had received repeated treatment elsewhere, and who were already in the late stages or the terminal phase of the disease when we first saw them; and (3) that as yet no analysis has been made of the survival rates of our patients on the basis of our clinical classification.

Our experience reveals that in two clinical settings in patients with Hodgkin's disease the combined therapy of 0.4 mg./kg. of HN2 (or 0.12 mg./kg. of [illegible]

compression with impending or present paraplegia. In the vena cava syndrome, full dosage increments of from 200 to 300 roentgens (air) should be administered up to a total dose of 2000 to 3000 r (air) to opposing anterior and posterior mediastinal fields This can be done without fear of further compression, provided it is preceded by the treatment with HN2 Alleviation of the classic symptoms and signs of this syndrome begins within 12 hours after initiation of such chemoradiotherapy and is complete within 2 or 3 weeks in over 75 per cent of patients

In a recently completed study of the occurrence and treatment of paraplegia due to Hodgkin's disease we found 50 instances of paraplegia in 1583 patients with the disease, these instances were associated almost exclusively with epidural Hodgkin's disease[7,8] More significant was the finding that excellent restoration of sensation and motor function, along with the disappearance of paraplegia, was achieved in 50 per cent of those patients treated by radiation therapy alone, and in 90 per cent of those patients treated by combined chemoradiation therapy[7,9] These findings in the largest single group of paraplegic Hodgkin's disease patients reported to date seem to negate effectively the need for the commonly recommended method of treatment, that is, laminectomy alone, or laminectomy and radiotherapy.

After careful neurological examination and localization of the lesion, the patient is given 0.4 mg./kg of HN2 I.V., and immediately afterward is started on 250 kv X-ray therapy to vertebral fields encompassing the entire site of neurological involvement Full increments of from 200 to 300 r (air) have been delivered to a total dose of 2000 to 3000 r (air) Pretreatment with HN2 prevents further compression of the spinal cord by the initial doses of radiotherapy[7-9]

Certain it is that much remains to be done to improve our early diagnosis of patients with Hodgkin's disease, our treatment techniques and, perhaps most of all, our philosophical approach to the treatment of these patients

In no sphere of cancer medicine is there such an appallingly nihilistic philosophy among physicians and surgeons, and even among radiologists, as there is in the treatment of patients with malignant lymphomas Yet we see swift and even enthusiastic treatment by radical surgery of cancers of the lung, pancreas, stomach, soft parts, and bone, to name a few, whereas the total

experience in terms of 5-year survival rates for *all* such patients hovers somewhat below 10 per cent!

Let us be critical of ourselves, of our present methods of treatment, and of our instructions to our colleagues and students, and impart a *dynamic* philosophy in treatment Let us continue to search out the past experiences, the natural history, and the vagaries of the biological activity of Hodgkin's disease while we press forward in our search for better radiotherapeutic and chemotherapeutic measures for this disease.

In the meantime, let us continue to prevail on our medical colleagues to attempt diagnois of Hodgkin's disease early, without temporization and guesswork, so that the therapeutic means now available to us can be used forthwith and to the best advantage of the patient.

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TWO-MILLION-VOLT X-RAY THERAPY OF HODGKIN'S DISEASE

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We have used X rays produced at two million volts for the treatment of lymphoma since 1949.[1] Several physical and clinical advantages result from the use of this higher-energy radiation in local and regionalized cases. The relatively greater penetration, as indicated by its half-value layer (HVL) of 12 5 mm copper, allows the administration of sufficient radiation to the deeper tumor-bearing regions. Untoward skin effects are almost completely avoided both because of this penetration and because of the fact these high-energy rays achieve their maximum ionization just below the radiosensitive skin[2] The threshold skin erythema dose with 2-million-volt X radiation is about 3000 r when delivered by the divided-dose technique, as compared with less than 1000 r for conventional deep-therapy rays. In addition, megavolt rays are known to be less strongly absorbed by bone and other high-atomic-numbered components and thus have a lesser effect on the blood-forming organs for the same soft-tissue dose.

The Van de Graaff electrostatic X ray source used in this work is a constant-potential unit delivering 80 r/min, measured at 1 meter. The usual treatment distance of 125 cm allowed a portal 35 cm × 35 cm. in size and made possible the treatment of most regions with a single field carefully shaped so as to shield normal structures such as the eyes, teeth, lungs, or genitalia The focal spot on these units is usually under 3 mm in diameter and results in a sharply defined X-ray beam These factors have allowed us to treat most of our lymphoma cases through two portals, one anterior and one posterior, with minimum tumor doses from 2400 r to 3000 r delivered to the primary lesion and the adjacent lymphatics, regardless of the location Conventional deep-therapy equipment cannot readily provide portals of the dimensions desirable for lymphoma therapy and may elicit excessive skin and systemic reaction if adequate deep irradiation is delivered to such large fields

The histological classification of lymphomas used in this study and the number of cases treated are shown in FIGURE 1 This report covers four series of cases followed for a period of three to five years for all types of lymphoma (FIGURE 2). The 64 cases reported using the 2-million-electron-volt (Mev) X ray were treated at Massachusetts Institute of Technology (M I T), between 1950 and 1952 and were patients for whom one of us (H F H) cared as a staff member of the Lahey Clinic, Boston, Mass Another group of 181 cases was composed of patients also treated by H F H between 1939 and 1942, 200-kilovolt-peak (kvp) X rays were used The third series of 63 cases was treated with 200-kvp and 400-kvp therapy at Los Angeles Tumor Institute,

* Fellow of the Los Angeles Tumor Institute

HISTOLOGICAL CLASSIFICATION	NUMBER OF CASES	RELATIVE INCIDENCE
HODGKINS	28	43.7%
RETICULUM CELL	7	10.9%
LYMPHOMA-UNCLASSIFIED	10	15.6%
LYMPHOSARCOMA	10	15.6%
MALIGNANT LYMPHOCYTOMA	1	1.5%
MACROFOLLICULAR LYMPHOMA	8	12.5%

FIGURE 1 Lymphoid tumors

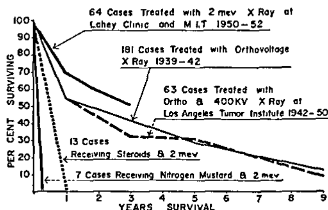


FIGURE 2 Lymphoid tumors, results of treatment—all stages Orthovoltage refers to treatment at 250 kilovolts or less

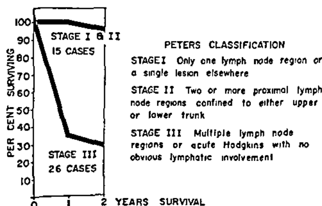


FIGURE 3 Results of treatment by stages, based on 41 cases treated with 2-Mev X rays at the Los Angeles Tumor Institute, 1954 to 1956

Los Angeles, Calif The fourth series of 479 verified cases of Hodgkin's disease (FIGURE 3) was from the California Tumor Registry and was treated between 1942 and 1953.

It is apparent from our study (FIGURE 3) that the Stage I and Stage II cases of lymphoma, in which the disease is local or regional, provide a much better opportunity for prolonged survival It is also evident that the radiation dosage is important in the Stage I and II cases since, in about 16 per cent of our cases, there has been local recurrence of the disease within the treated field when 2400 r was administered to the tumor site within a period of three weeks Although [illegible]

yields better results both in the first two years after treatment and in the five-year survival rate

Like most other institutions treating this disease, we have used nitrogen mustard and steroid compounds Some temporary palliative results have been obtained from the use of nitrogen mustard, although patients are sickened, and their blood is invariably adversely affected by it, especially as regards the white cell and platelet counts (FIGURES 4–6). The average survival of 7 cases of advanced generalized disease receiving nitrogen mustard combined with 2-Mev X rays was approximately four months. While most of these cases would have lived that long without the addition of nitrogen mustard, several obtained relief Patients receiving steroid compounds and 2-Mev radiation have lived longer and have experienced a better palliative result than with nitrogen mustard and radiation From our experience the radiomimetic drugs should be used only in the Stage III cases, and then only when the steroid compounds are ineffective

The histological classification of the lymphoid tumors is important as an [illegible]

experience (FIGURE 7) The results of treatment of these two types strongly supports a unicentric origin of the disease, it may be that many of our failures in the more rapidly growing tumors are due to the fact that we do not irradiate enough of the adjacent lymphatic system

Lymphatic anatomy and circulation to a large extent determine the mode of lymphoma spread The thoracic duct is the major trunk of lymphatic circulation for the entire body except portions of the head, neck, upper extremities, and thorax Constant centripetal flow of lymph is maintained by capillary filtration pressure and muscle action At the junction of the thoracic duct and jugular vein the pressure differential is 2 to 3 cm of water. Spread of tumor via lymphatics is usually a matter of emboli Retrograde permeation will occur when regional lymph nodes are completely filled with tumor In our series, when the head, neck, and mediastinum have been involved, we have treated the thoracic duct down to the diaphragm, but only in the abdominal cases have we treated the cisterna chyli One expects from physiology

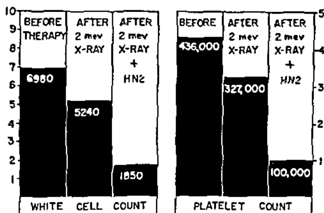


FIGURE 4 Lymphoid tumors, generalized effect of therapy with 2 Mev X rays and nitrogen mustard (HN2) on blood-formed elements

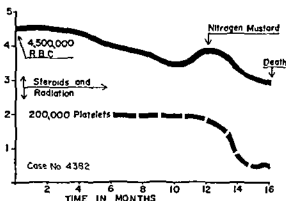


FIGURE 5 The relative effect of therapy and disease on blood formed elements; red blood count and platelets

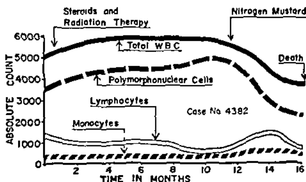


FIGURE 6 Lymphoid tumors, the relative effect of therapy and disease on blood formed elements—white blood cells

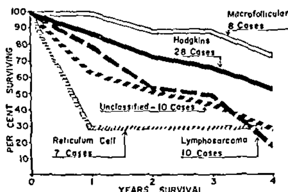


FIGURE 7 Lymphoid tumors, four year results of treatment with 2 Mev X rays by histological types

that the lymphatic flow would be from the abdomen superiorly into the venous system However, it is probable that in some cases there must be a reversal of the normal flow, for it has been well demonstrated that in 90 per cent of the lymphomas the thoracic duct is involved Charles Benz and William H Brown of Presbyterian Hospital in Los Angeles, Calif (personal communication), inadvertently injected the thoracic duct, and this is the only demonstration we have seen showing the cisterna chyli and thoracic ducts (FIGURE 8) After reviewing the lymphatic flow shown by this radiograph we believe treatment of this area is necessary and, from now on, we plan to include it in our treatment field



FIGURE 8 Lymphoid tumors, cisterna chyli and thoracic ducts

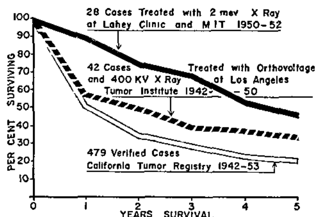


FIGURE 9 Hodgkin's lymphoma, five-year results of treatment—all stages

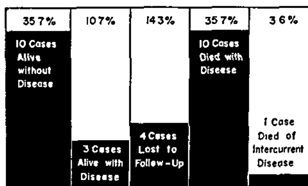


FIGURE 10 Hodgkin's lymphoma, results of treatment by stage and class Based on 28 cases treated with 2 Mev X rays at Lahey Clinic and at M I T, 1950 to 1952

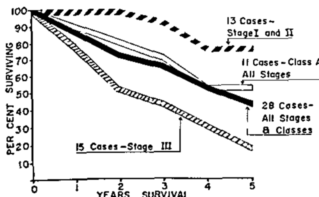


FIGURE 11 Hodgkin's lymphoma, results of treatment by stage and class Based on 28 cases treated with 2-Mev X rays at Lahey Clinic and M I T, 1950 to 1952

The primary interest in this presentation is Hodgkin's lymphoma (FIGURE 9). A comparison is made of the results of several treatment methods in several institutions, the survival being determined from the date of biopsy and without reference to symptoms or presence of nodes. The California Tumor Registry, Berkeley, Calif., reports the results of therapy between 1942 and 1953 on 479 verified cases by the 26 reporting centers, regardless of the method used. The cases reported from the Los Angeles Tumor Institute were treated by either 250-kvp or 400-kvp X ray and received a dose of radiation varying from 2000 r to 2400 r to the primary tumor, the radiation being delivered in approximately 21 days. The 28 cases treated in the Lahey Clinic and the M.I.T. patients received a tumor dose of at least 2400 r delivered in 18 to 24 treatment days and were treated with what we considered a large enough port to cover adequately the primary disease and the adjacent lymphatics. The complications of this latter treatment were not severe except in one case. This was the first Hodgkin's case treated with 2-Mev radiation by us, the multiple adjacent fields may have produced overlapping of portals. A year later cord damage occurred following trauma at the region of possible overlap. Because of this, single fields shaped with special care to avoid or stagger overlap have since been routinely used. Muscle and fat fibrosis is occasionally seen 6 to 8 months following treatment.

The follow-up on these patients treated with 2-Mev X rays (FIGURE 10) indicates that, of 28 cases, 10 patients are alive without disease, 10 are dead with disease, and 3 are alive with disease, with 4 cases lost to follow-up, and 1 case dead of intercurrent disease. It is of further interest to tabulate the results according to stages (FIGURE 11).



FIGURE 12

FIGURE 13

FIGURE 14

One of the most important aspects of our radiation procedure is the close

posterior projections (FIGURES 12, 13, and 14).

4 weeks The apparent relative prolongation of symptom-free life by this treatment method is noted As expected, with all types of radiation therapy, markedly better results are obtained in Stage I and Stage II cases On the basis of our experience we urge early biopsy of suspected cases and recommend immediate institution of adequate radiation without the use of radiomimetic drugs

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THE SCOPE OF CHEMOTHERAPY IN HODGKIN'S DISEASE

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We shall assume, for the purpose of this discussion, that Hodgkin's Disease is a form of cancer linked most closely with the leukemias and the lymphomas.

Acknowledgment must be made of the devoted and inspired clinical efforts of a small group of clinicians in this country who have been responsible for prolonging the life of patients with Hodgkin's disease and studying critically every therapeutic possibility emerging from the laboratory that might be employed for the good of the patient Representative of such men are some of the participants in the conference on which this publication is based, including Lloyd F Craver and Antonio Rottino.

This paper could not have been written fifteen years ago A large part of it is the product of research of the last few years and of work that is presently in a high state of activity in a number of laboratories and clinics throughout the world.

The discovery of the anti-Hodgkin's properties of the nitrogen mustards, now a part of medical history, provided justification for the hope that more effective and more specific chemical agents against the disease could be found. The list of chemical substances, hormones, and antibiotics that have been found to exert a temporary but important control of the course of Hodgkin's disease in a large percentage of patients is now long enough to permit a retrospective glance and to make plans for future research on the basis of proved accomplishment by chemical substances alone.

We shall consider here a few of the problems concerned with the formulation of research programs devoted to the search for substances that may more effectively control the Hodgkin's process and, we may hope, destroy it completely without harm to the patient We shall use the term chemotherapy broadly, to include the action not only of chemicals but also of plant extracts, hormones and hormone analogues, and antibiotics and bacterial products such as the polysaccharides Agents that have temporarily controlled the Hodgkin's process have been in large part (1) alkylating substances, the most potent of which have been the nitrogen mustards, TEM, and chlorambucil, (2) the hormone ACTH, and the corticosteroids, (3) antimetabolites, such as the folic acid antagonists, and, more recently, (4) antibiotics, such as Actinomycins C and D[1]

Our knowledge of the exact mechanism of action against Hodgkin's disease of all of these agents, or of any one of them, is insufficient to be used as a sound foundation for the creation of a rational program of synthesis of the ideal anti-Hodgkin's agent Although some of these substances were employed against Hodgkin's disease because of evidence, derived from experimental work, that they possessed a destructive power against the lymphocyte, or were capable of causing profound interference with hematopoiesis, all of these substances

have been used in an attempt to control other forms of cancer, and against some of these there has been some degree of success It will be more profitable, therefore, to depart from Hodgkin's disease, as such, for the next part of this discussion, and to treat that disability for the moment merely as one of a number of forms of cancer against which chemotherapy presently is being employed

Can the Present Agents Be Made More Effective?

Two outstanding deterrents to the more effective use of these chemotherapeutic weapons against Hodgkin's disease may be mentioned. The first has to do with the resistance of the tumor to the chemical compound to which it had once been sensitive; the second, with the toxicity of these chemicals

Resistance. It is true that every chemotherapeutic agent that has so far proved to be carcinolytic or carcinostatic in either the experimental animal or in man has encountered the resistance of the surviving tumor cells The phenomenon here appears to be similar to the acquired resistance of bacteria to antimicrobial agents. Theories based in part upon experimental evidence assume mutation of the surviving cells, a possible alteration in the permeability of the cell to the chemical agent, alterations in the utilization of the chemical, which may become a food for the cell instead of a poison and, finally, the development of alternate metabolic pathways by the cancer cell for the synthesis of nucleoprotein There has been discovered no simple method of overcoming this form of acquired resistance, despite many promising suggestions based upon laboratory experiments The one practical hope at the present time lies in the availability and utilization of another powerful anti-Hodgkin's agent to be substituted for the one to which resistance has been acquired Such a new agent must not be closely related in structure or biological activity to the previous one (for example, resistance to one folic acid analogue is exhibited against all folic acid analogues)

Toxicity It is always justifiable to hope that the action of analogues of a given carcinolytic agent may possess as much or even greater carcinolytic activity with greatly reduced toxicity This expectation has not been realized to any practical degree thus far The toxicity, in general, of most of the chemical agents has been manifested by bone marrow depression, alterations in the intestinal mucosa, and in other rapidly dividing cells, with great variation in involvement of parenchymal organs and the brain when larger dosages are employed If the bone marrow could be protected or replaced, much greater amounts of the anticancer chemicals could be administered A warning should be given, however, that even if we succeed in protecting or replacing completely the bone marrow in a given patient, the administration of very much larger amounts of the chemical agents may be productive of serious damage to the brain or to the viscera, this possibility must be kept in mind in the enthusiasm for bone marrow replacement The recent interest in bone marrow replacement has led to much animal and clinical investigation The possibility of removing some of his own bone marrow from a patient with cancer, storing it under optimal conditions, and returning it the the patient eventually, days after the administration of obviously toxic amounts of chemical substances, is now

being explored. At the present time there is still no easy source for large amounts of bone marrow from appropriate banks, although this problem may be solved by work presently in progress in a number of institutions Should such a practice become routine, it may be possible to destroy many forms of cancer by the use of the chemicals presently available, provided the amount of cancer is not too great

In the case of Hodgkin's disease, I doubt that permanent destruction of the Hodgkin's process may be accomplished by the use of the agents presently available, even if they can be used in doses several times those presently employed. I suggest that Hodgkin's disease, the leukemias, and the lymphomas differ from other forms of cancer in respect to the existence of cells situated in the interstices of the body that have the potentiality to form new tumor when acted upon by the cause of these forms of cancer Such cells may remain after all visible tumor masses are destroyed It is possible that two kinds of agents may be needed One substance, such as nitrogen mustard or chlorambucil, may be responsible for destroying large amounts of Hodgkin's tumor and suppressing further growth for varying periods of time. A second and perhaps unrelated substance may then be used to act destructively against viral agents, genes, or chromosomal fragments that may have the power to act upon normal cells and render them neoplastic

Rational Approaches to Chemotherapy of Hodgkin's Disease

The alkylating agents, called also polyfunctional cytotoxic agents, are thought by some to be responsible for enzyme inactivation, although a number of other theories have been propounded. There is a wave of pessimism concerning the value of synthesis of more agents in this class The important carcinolytic activity of the aromatic nitrogen mustards such as chlorambucil, particularly against Hodgkin's disease, gives encouragement to the synthesis of new alkylating agents The striking antileukemic effect of such folic acid antagonists as Aminopterin and methotrexate have stimulated search for other anticancer compounds based on the principle of antimetabolite action The folic acid antagonists prevent cells from using single carbon atoms for the synthesis of several amino acids and, in addition, prevent the use of these carbon atoms for building the purines and pyrimidines that are essential constituents of nucleic acids The value of 6-mercaptopurine in acute leukemia has emphasized the antimetabolite approach The largest number of rational programs are concerned with the search for nucleic acid inhibitors, including such substances as antagonists to pyrimidines, purines, thymine, and amino acids. It is true that the greatest amount of biochemical research points to nucleic acids as the very center of the cancer problem It should be emphasized that other vital components of cells, including lipids, carbohydrates, trace elements, and other minerals, should not be neglected in this search for anticancer agents

Is it possible to organize a rational approach to the chemotherapy of Hodgkin's disease? The exact nature and cause of Hodgkin's disease are not known Bostick has outlined evidence in favor of the virus etiology of Hodgkin's disease elsewhere in these pages The leading proponent of the theory that

a virus may play a part in the causation of the various forms of cancer is Wendell Stanley,[2] who has pointed out that it is obvious that the viruses, cancer, genes, and life itself are tied together by a whole series of relationships; furthermore, that viruses can act as genes, and genes as viruses, under certain circumstances Stanley has stated that it is clear "that viruses can cause cancer and that viruses are structures at the twilight zone of life, partaking both of life and molecular properties" In a series of ingenious and logical steps, he has pointed out that the same "stuff of life" holds for viruses as well in the light of recent discoveries that special treatment of tobacco mosaic viruses yielded a nucleic acid preparation possessing virus activity He conceives of a thousand-unit polynucleotide linear chain consisting of a coded repeat of only four different components—adenine, guanine, cytosine, or uracil (thymine, in the case of desoxyribonucleic acid)—the code "for every bit of life on earth and in the sea" Stanley postulates that, when a normal cell becomes a cancer cell, there is undoubtedly a change in this structure within the cell, and he points out that the incorporation of 5-bromouracil into a bacterial virus in place of thymine results in the production of the highest percentage of mutants ever recorded What is of interest here is that Stanley comes to the importance of antimetabolites in the chemotherapy of cancer from a chain of evidence derived from studies of the chemistry of genes, viruses, and bacteria to reach a point shown during the past few years by cancer chemotherapists to be the center of interest in the chemotherapy of cancer in man. Here is an important meeting ground of cancer chemotherapy and virus research

I suggest, then, that whether Hodgkin's disease is shown to be caused by a virus, or if it should be possible to demonstrate the existence of a transduction process in man, the role of chemotherapy in Hodgkin's disease will remain the same. We must recall that Paracelsus, more than 400 years ago, spoke of a specific chemical taken into the body wherewith to drive out all venoms of a specific disease This concept was put in scientific language by Paul Ehrlich, who founded the science of chemotherapy, a term that he coined, not to imply that chemistry was to be utilized in the treatment of the disease, as in iatrochemistry, but rather in the destruction of the specific disease-producing living

against the formation of the nucleic acids responsible for the perpetuation either of the viral cause of Hodgkin's disease or for igniting the activity of viruses, genes, or chromosomal particles required for the transformation of normal precursor tissue cells into Hodgkin's tumor, or for the multiplication of surviving tumor cells.

The Cancer Chemotherapy National Program

The Cancer Chemotherapy National Program (CCNP), inaugurated a little more than three years ago after Congressional action the year before, now represents the largest voluntary cooperative medical program ever attempted in peacetime in this country It has a budget at present representing nearly one half that of the entire total of the National Cancer Institute of the Public

Health Service at Bethesda, Md. This program has grown enormously during the past three years under the guidance of the Cancer Chemotherapy National Committee, which is composed of representatives of the American Cancer Society and The Damon Runyon Memorial Fund for Cancer Research, Inc., both of New York, N. Y., the National Cancer Institute, the Atomic Energy Commission, the Food and Drug Administration, and the Veterans Administration, all of Washington, D. C., under the administrative leadership of Kenneth M. Endicott, Chief of the Cancer Chemotherapy National Service Center in the National Cancer Institute.[3] The two major purposes of the CCNP are the acceleration of research in chemotherapy of cancer and the perfection of

addition, large cooperative programs in the several areas of interest, such as organic chemistry, screening methodology, pharmacology, biochemistry, endocrinology, and clinical investigation have made possible rapid expansion in all areas. Of great importance has been the participation of industry, which has not only placed its many thousands of compounds and antibiotic beers at the disposition of the national program, but has also accepted contracts for basic research of importance to chemotherapy, as well as for the production of chemicals and antibiotics, the testing of agents for anticancer effects, and the development of new methods of importance in the many areas concerned with the discovery, production, purification, and preparation of a chemotherapeutic agent for application to man.

Paralleling the conduct of as much basic research as scientists have desired to carry out has been a gigantic empirical program which, during this past year under the direction of the National Center, permitted the testing against transplanted tumors in the mouse of over 45,000 chemicals and antibiotic beers Unpopular as such empirical programs may be with many investigators, the history of science is replete with examples of important advances originating from empirical methods applied under the direction of trained observers It is possible, therefore, that curative agents for Hodgkin's disease will arise from these empirical programs, or that chemicals very much more effective and less toxic than those presently available may be so discovered Two classes of potential chemotherapeutic agents will be mentioned because of their possible bearing on the treatment of Hodgkin's disease

Hormones and hormone analogues It is estimated that there are at least 4000 analogues of known hormones that have been synthesized by industry and have not yet been studied against any form of cancer in man, including Hodgkin's disease The striking temporary effects produced by corticosteroids and ACTH justify the careful search for an anti-Hodgkin's effect of these synthetic hormonelike chemicals. The development of testing devices in the laboratory to give close correlation with activity against Hodgkin's disease in man will make the task very much easier

Antibiotics A number of antibiotics have been shown to have anticancer effects, some of striking degree, against tumors in experimental animals and, in a few instances, in man Azaserine, derived from a *Streptomyces* species,

is an antagonist to glutamine in the pigeon-liver system and so interferes with purine biosynthesis Azanorleucine* (DON) interferes with the *de novo* synthesis of purines It was isolated also from a species of *Streptomyces* and is similar in structure to azaserine. There are no records of extensive studies of the action of these compounds against Hodgkin's disease. Actinomycin C and a material related chemically and biologically to it, Actinomycin D, are at this

most powerful agent by weight against a number of transplantable tumors in mice that I have encountered, and it has produced actual "cures" of mouse mammary adenocarcinomas after prolonged administration. In man, it is the first agent to have a profound effect upon Wilms's tumor metastases to the lung Because of its powerful antileukemic and antilymphosarcoma effect in mouse tumors, it would be of great interest in combatting Hodgkin's disease if it were not for its high degree of toxicity. Only very small doses can be tolerated, and these are too small to have a lasting effect upon Hodgkin's disease. The discovery of these important, although temporary, anticancer effects of antibiotics in man has produced justification for a large-scale production and study of antibiotics The pharmaceutical industry has taken over this opportunity, in part with the aid of contracts from the Cancer Chemotherapy National Program It is confidently to be expected that from such mass production of antibiotics and the search for anticancer activity, antibiotics of great value as chemotherapeutic agents in cancer will be discovered

Combination therapy If laboratory methods could be constructed for the accurate evaluation of combinations of chemical agents that might be used together, in sequence, or in combination with radiotherapy, the task of choosing the proper method of treatment in Hodgkin's disease would be greatly simplified Lacking such laboratory aids, we may ask those clinical investigators who are prepared to carry out such studies whether a retrospective study of

of compounds, added to antibiotics in proper dosage so that they may be administered with safety Understandable reluctance to embark upon such studies without more knowledge concerning mechanism of action will make progress in this direction in the treatment of human Hodgkin's disease slow, indeed

The Potentiation of Radiotherapy by Chemical Substances

In many institutions a search has been made for substances that could

* This compound is 6-diazo-5-oxo-*nor*-L-leucine

Health Service at Bethesda, Md. This program has grown enormously during the past three years under the guidance of the Cancer Chemotherapy National Committee, which is composed of representatives of the American Cancer Society and The Damon Runyon Memorial Fund for Cancer Research, Inc., both of New York, N Y, the National Cancer Institute, the Atomic Energy Commission, the Food and Drug Administration, and the Veterans Administration, all of Washington, D C, under the administrative leadership of Kenneth M Endicott, Chief of the Cancer Chemotherapy National Service Center in the National Cancer Institute[3] The two major purposes of the CCNP are the acceleration of research in chemotherapy of cancer and the perfection of means of communication of the results of research without delay to investigators in all countries. Greatly increased support to individual investigators, groups, and institutions has been made available for basic or applied research In addition, large cooperative programs in the several areas of interest, such as organic chemistry, screening methodology, pharmacology, biochemistry, endocrinology, and clinical investigation have made possible rapid expansion in all areas. Of great importance has been the participation of industry, which has not only placed its many thousands of compounds and antibiotic beers at the disposition of the national program, but has also accepted contracts for basic research of importance to chemotherapy, as well as for the production of chemicals and antibiotics, the testing of agents for anticancer effects, and the development of new methods of importance in the many areas concerned with the discovery, production, purification, and preparation of a chemotherapeutic agent for application to man

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of Hodgkin's disease to warrant the conclusion that in this direction lies one complete and satisfactory solution of the problem of Hodgkin's disease

References

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these observations to Hodgkin's disease is now under way in our own institution. In 1953 we reported the conversion of a radiosensitive rhabdomyosarcoma to an extremely radiosensitive tumor after two months of treatment with a folic acid antagonist, the 4-amino-9,10-dimethyl folic acid. More recently, with the collaboration of G. J. D'Angio, J. E. Pollock, A. E. Evans, R. Toch, and other colleagues, we have observed the striking potentiation of radiotherapy by Actinomycin D given intravenously. No agent studied by us has yielded such potentiation of radiotherapy effect. This has been noted particularly against several forms of sarcoma not ordinarily radiosensitive, such as the rhabdomyosarcoma. At the present time we are studying the potentiation of radiotherapeutic effect on Hodgkin's disease, not only on previously untreated Hodgkin's disease, but also after resistance to radiotherapy alone has been reached. This represents one more way in which methods of proved value, such as radiotherapy, may be made more effective against Hodgkin's disease, and it provides one additional hope for increased survival with the use of the therapeutic weapons presently available.

Conclusions

The scope of chemotherapy in the treatment of Hodgkin's disease will increase greatly in the immediate future. Rational programs of synthesis based upon knowledge of mechanism of action of those chemical compounds presently effective in causing temporary control should yield compounds worthy of clinical investigation. The vast undertaking of the Cancer Chemotherapy National Program studies will certainly bring to the clinical investigator increased numbers of materials for study. Expert use of the compounds presently available may result in far better clinical results if problems such as toxicity, bone marrow depression, resistance, and optimal combinations for therapy can be solved. Research programs not designed primarily to solve problems concerned with the chemotherapy of Hodgkin's disease alone may be of fundamental importance. A prime example is the use of bone marrow, either in cellular form or in chemical fractions given intravenously to repair the bone marrow damage caused by the use of larger amounts of anti-Hodgkin's chemotherapy. Finally, the study of the possible potentiation of radiotherapy by the action of such chemicals as Actinomycin D may cause far greater and more permanent destruction of the Hodgkin's tumor with no increase in the

to the patient with Hodgkin's disease, on an empirical basis, of chemical agents, hormones, or antibiotics considered worthy of study against any form of cancer in man on the basis of obviously imperfect methods of laboratory selection. There is sufficient evidence available from research in the various areas of cancer chemotherapy and from achievements in the chemotherapy